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EFFECT OF VITAMIN E DEFICIENCY ON THE LEVELS OF CERTAIN PHOSPHOLIPIDS IN RAT LIVER AND TESTES SUBCELLULAR MEMBRANES

by

© MARGARET E. CORNS

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE.

DEPARTMENT .....Pediatrics.....

EDMONTON, ALBERTA

FALL, 1980

THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled ..Effect.of.vitamin.E.deficiency.on.the.levels.of.certain..... phospholipids.in.rat.liver.and.testes.subcellular.membranes..... submitted by ..Margaret.E..Corns..... in partial fulfilment of the requirements for the degree of Master of Science.

.....
Supervisor

.....

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Date ...Sept 4, 1980.....

The science of life is a superb and
dazzlingly lighted hall which may
be reached only by passing through
a long and ghastly kitchen.

An Introduction to the Study
of Experimental Medicine

Claude Bernard
1813 - 1878

DEDICATION

THIS WORK IS DEDICATED TO
MY SON DANIEL MATTHEW WHO
HAS GIVEN ME MUCH JOY.

ABSTRACT

The present study was undertaken to determine whether vitamin E deficiency results in decreased levels of any of the three principal phospholipids of rat liver or testes subcellular membrane, namely phosphatidyl choline, phosphatidyl ethanolamine or diphosphatidyl glycerol. Twenty weanling rats were allocated to an experimental group and received a vitamin E deficient powdered diet based on 10% corn oil. Twenty weanling rats served as controls and received a diet identical to that of the experimental group except for the addition of 250 mg dl-alpha-tocopherol acetate per kilogram diet.

During the first 3 weeks of the study the 2 groups of rats were pair fed. They were then allowed to feed ad libitum. Except for weeks 19 and 20, when the experimental group averaged less than the control group, there was no significant difference in mean weight between the 2 groups. The animals were sacrificed over a 4 week period, beginning at the twenty-fourth week of the feeding trial. Blood was collected for hematocrit and serum vitamin E determinations. Pooled samples of liver and testes tissue were frozen for subsequent tissue vitamin E determinations. Subcellular membrane pellets were prepared from pooled samples of liver and testes tissue and the resulting lipid extracts were frozen for phospholipid analyses. Deterioration of the diphosphatidyl glycerol component was noted over time, and a second set of subcellular membrane pellets and lipid extracts had to be prepared from previously frozen, pooled tissue. The second lipid extracts were chromatographed within 24 hours of extraction. Individual phospholipids were quantitated by phosphorus assay.

Serum and tissue vitamin E determinations revealed that there was a significant difference in vitamin E status between the 2 groups,

and that the experimental group was vitamin E deficient. The magnitude of the deficiency was questioned, because the tissue vitamin E levels of the experimental group were not as low as those reported by some investigators, and because there was no difference in tissue weight between the 2 groups. Analysis of the subcellular membrane fraction revealed that there were no differences between the 2 groups with respect to protein level or the amount of membrane isolated. Electron microscopic examination of the membranes with a negative staining technique showed no differences in ultrastructure or membrane density, although some photographs showed vitamin E-deficient mitochondria with loss of matrix. There were no differences between the 2 groups in any of the 3 subcellular membrane phospholipids evaluated. It was concluded that, under the conditions of this feeding trial, vitamin E deficiency does not result in lowered levels of rat liver or testes subcellular membrane phosphatidyl choline, phosphatidyl ethanolamine or diphosphatidyl glycerol.

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LIST OF ABBREVIATIONS

DPG	Diphosphatidyl Glyerol
IM	Inner Membrane (of mitochondria)
OM	Outer Membrane (of mitochondria)
PC	Phosphatidyl Choline
PE	Phosphatidyl Ethanolamine
PUFA	Polyunsaturated Fatty Acids
RBC	Red Blood Cell (erythrocyte)
SM	Subcellular Membrane

I. INTRODUCTION

Red blood cells of vitamin E-deficient rats have been shown to hemolyze following in vitro exposure of the red blood cells to hydrogen peroxide. Prior to this hemolysis, a decrease in the level of both polyunsaturated fatty acids and of phospholipids, primarily phosphatidyl ethanolamine, can be demonstrated. These changes are accompanied by an increase in fluorescence, indicative of lipid peroxidation. Alterations in the shape of rat red blood cell membrane also have been reported in association with vitamin E deficiency. Similar effects of vitamin E deficiency have been observed in membranes other than the red blood cell membrane. In rat mitochondrial and microsomal membranes an increase in fluorescence has been found to be inversely proportional to dietary vitamin E, while slightly decreased levels of rat liver mitochondria total phospholipid have been reported in association with vitamin E deficiency. Such effects have been attributed to the effects of lipid peroxidation. It is theorized that vitamin E acts in vivo as an antioxidant by preventing lipid peroxidation, the latter being a deteriorative process which occurs in membranes when the lipids in the membrane react with oxygen and produce unstable free radicals followed by semi-stable peroxides. While peroxidation has not been demonstrated in vivo, electron microscopic examination of human as well as duck subcellular membranes have demonstrated that ultrastructural changes occur as a result of vitamin E deficiency. Such observed changes include mitochondrial swelling, loss of matrix and fragmentation of cristae. As well, a decrease in the thickness of the outer mitochondrial membrane and an increase in the thickness of both the inner mitochondrial and endoplasmic reticulum membranes have been observed.

The objectives of the present study were (1) to measure the levels of liver and testes subcellular membrane phosphatidyl choline, phosphatidyl ethanolamine and diphosphatidyl glycerol in a group of vitamin E-deficient rats, (2) to compare them to a group of control rats who received an identical diet except for the addition of vitamin E, and (3) to relate any detectable change in the ultrastructure of the subcellular membranes to changes in these phospholipid levels.

II. LITERATURE REVIEW

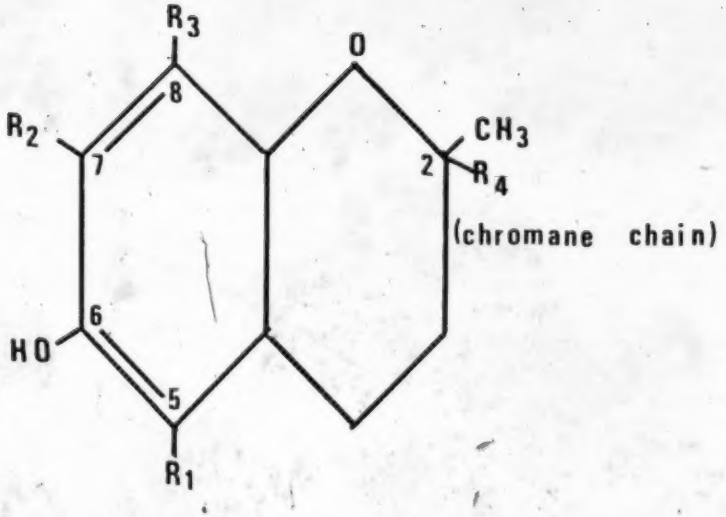
A. Introduction

Vitamin E is a collective term used to describe any one of 8 forms of naturally occurring tocochromanols. These forms include alpha, beta, gamma and delta tocopherols, and the four corresponding tocotrienols (13, 109, 127). The biological activity of the tocochromanols is derived from the chromane chain (see Figure 1) and is destroyed by photo-oxidation (52, 78). One International Unit of vitamin E is equivalent to 1 mg of dl-alpha-tocopherol acetate. Alpha-tocopherol[1], which is commonly designated as "vitamin E", has a biological activity of 135% relative to beta (50%), gamma (10%) and delta (1%) tocopherols (31, 78).

Manifestations of vitamin E deficiency differ from species to species, and include muscular dystrophy[2] in herbivorous animals (rabbit, sheep, cattle), encephalomalacia of chicks[3] and hemolytic anemia in humans (58, 61, 98, 122). Restoration of reproductive capacity with doses of vitamin E is a phenomena peculiar to the vitamin E-deficient rat (35, 47). Severe deficiency of vitamin E is rare in man, and until recently, it was assumed that day-to-day inadequacies of intake could be offset by the large capacity of the body to store vitamin E.[4] Recently, Machlin et al (104) have demonstrated in guinea pigs, that, under conditions of vitamin E depletion, there is little release of vitamin E from fat depot, and speculate that a person could be vitamin E-deficient in spite of having a high stored reserve of the vitamin.

The basic requirement for vitamin E can be supplied by a balanced diet (35). Rich sources of vitamin E include colostrum for the neonate (109, 117) and beyond this age, vegetable oils, wheat germ, and

Figure 1. Structure of Vitamin E compounds(78).



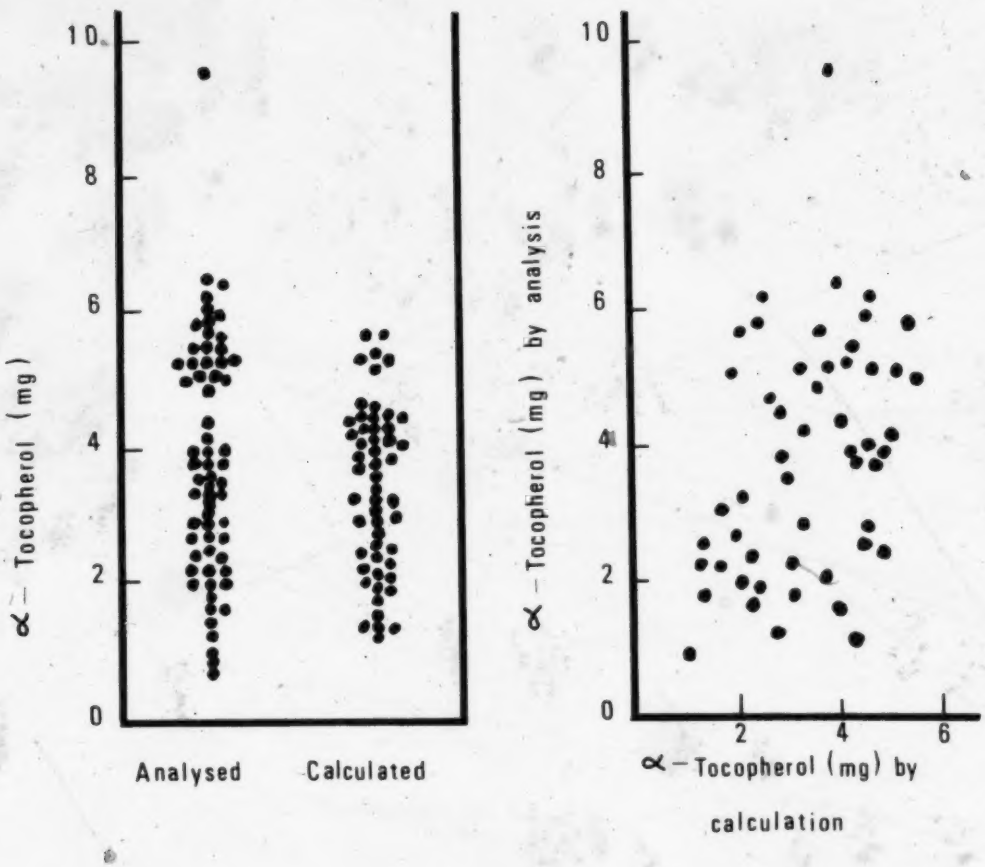
TOCOLS R_4: $CH_2(CH_2CH_2\underset{\displaystyle CH_3}{\underset{|}{C}}HCH_2)_3H$

TOCOTRIENOLS R_4: $CH_2(CH_2CH{=}\underset{\displaystyle CH_3}{\underset{|}{C}}CH_2)_3H$

salad greens, especially leaf lettuce (109). However, there is a possibility that a significant percentage of human beings may have a borderline deficiency of vitamin E in view of some present day lifestyle changes, and the consideration that good uptake and absorption does not necessarily ensure adequate tissue-content of vitamin E (115). Studies using mice and rats indicate that the need for dietary vitamin may increase in obesity (18, 162). Low plasma tocopherol levels have been reported among users of oral contraceptives (157). People with sickle cell anemia may have an increased need for vitamin E (29). Tocopherols are destroyed during processing and industrial cooking (109, 115) which is significant in view of the trend towards large-volume restaurant sales and the use of convenience foods in the home (109). Smith et al (149) analyzed the vitamin E content of the diets of 10 normal, young subjects and 40 hospitalized patients, and concluded that the majority of British diets have a vitamin E content less than the recommended (British) intake of 5 mg per day despite being generally satisfactory in calories, protein and fat content. There was no correlation between the analytical results of the food and the values obtained by calculating the vitamin E content from food tables, even though both sets of data occupied the same range of values (see Figure 2). This likely was because the levels of vitamin E in foods vary greatly, depending on growth conditions for plants and feed for animals (109).

The use of mega-doses of vitamin E recently has become common in a substantial segment of the population (3, 35, 50). Proponents of vitamin E therapy claim it to be effective in the treatment of a wide variety of clinical conditions (147). Vitamin E may be beneficial in the therapy of Raynaud's disease (65), intermittent claudication (65), retrolental fibroplasia and hemotylic anemia of the newborn (58, 61, 98, 122),

Figure 2. Alpha-tocopherol contents of a sample of British diets as obtained by direct analysis and by calculation from food tables(149).



and may reduce hyperbilirubinemia in preterm infants (62).

B. Physiology of Vitamin E

Vitamin E is fat soluble and, after absorption, travels via the thoracic duct to the blood stream (56, 87, 99). Recent studies suggest that vitamin E is transported in the blood bound to a specific alpha-tocopherol-binding very low density lipoprotein (132).

Gallo-Torres (56) demonstrated that bile is necessary for the intestinal absorption of vitamin E, but Losowsky and associates (99) argue that this is likely secondary to its effect on the absorption of fat. The presence of fat is essential, not only as a source of vitamin E, but for its absorption (105). Once a dietary fat level of approximately 5% is present, increasing the percentage of fat in the diet does not result in increased absorption of vitamin E. As the level of dietary vitamin E increases, there is a corresponding decrease in the percentage of vitamin E absorbed (99).

Bieri and Poukka-Evarts (18), while investigating the effect of altered plasma lipids on tissue vitamin E levels, found that in hyperlipemic, obese rats the plasma total lipids and vitamin E levels were 3 times greater than in the control rats. Conversely, hypolipemia was associated with low levels of vitamin E. The obese rats were found to have more total body tocopherol than did the controls, but their tissue vitamin E levels were lower.

MacMahon and Neale (105) have demonstrated that vitamin E is poorly absorbed in patients with steatorrhea of various causes, such as cystic fibrosis and celiac disease. Premature infants also have a very limited capacity to absorb vitamin E, and have difficulty achieving vitamin E sufficiency even when receiving a supplement (58, 61, 98).

The use of medium-chain triglycerides in infant formula has not been found to improve vitamin E status (169). Tocopherol absorption increases with gestational age. Infants at 3, 6 and 10 weeks of life reveal progressively greater capacities to absorb vitamin E (111). A positive correlation exists between age and vitamin E absorption up to the age of 10 years (110). Likely, this is because premature and full term infants have decreased bile salt production and secretion relative to older children (140).

In studying the effect of age, sex and tissue specific differences on tissue vitamin E levels, Weglicki and associates (166) found that the livers of both mature and senescent female rats had higher concentrations of vitamin E when compared with males of both age groups. When compared with younger, mature rats, senescent male and female rats had significantly greater tocopherol levels in liver, heart and adrenal tissue, but this was not found in brain tissue. In female rats, the vitamin E requirement for reproduction increases with age (2).

When isotopically labelled vitamin E is administered orally to rats, adrenal and spleen tissue take up the most radioactivity on the basis of tissue weight and lipid composition (112). Liver tissue, however, accepts the greatest percentage of the dose, while testes takes up the smallest portion of tocopherol, an interesting phenomena in view of the known high requirement of testes tissue for vitamin E (26). The intracellular distribution of tocopherol varies with the tissue type. While liver microsomal tocopherol is 4 times more concentrated than either nuclear or mitochondrial vitamin E, the mitochondria of spleen have 3 times the concentration of tocopherol compared to microsomes (112). Pearson and Barnes (127) have demonstrated that each tissue can selectively absorb among the 8 tocochromanols and that some tissues are able to metabolize one form of

tocopherol into another form.

Studies of both human and rat blood have shown that all red blood cell (RBC) vitamin E is present in the membrane component of the cell (31, 148). Plasma tocopherol is in a dynamic equilibrium with RBC tocopherol, and exchange of vitamin E takes place at a rapid rate in a process which resembles the incorporation of fatty acids or cholesterol into RBC's (148). Kayden et al (85, 86) have demonstrated that plasma vitamin E levels determine the concentration of RBC vitamin E and that the ratio (approximately 0.21 for humans and 0.39 for rats) falls within a relatively narrow range over a wide range of plasma concentrations. According to Bieri (19), the RBC:plasma vitamin E ratio is affected by the plasma lipid level (and perhaps other factors), and may explain why there is often a poor correlation between plasma vitamin E and hemolysis. Increasing the plasma lipids up to 3 times their normal value shifts the ratio from 1:5 to as low as 1:20.

C. Postulated Role of Vitamin E: The Antioxidant Hypothesis

In 1952, Rose and György (138) reported that the RBC's of vitamin E-deficient rats were hemolyzed by dialuric acid. Vitamin E, whether fed to the rats, or incubated with the cells in vitro, was found to protect the RBC's from hemolysis. Catalase was found to inhibit the hemotytic effect of dialuric acid, thus it was concluded that the hemolytic action of dialuric acid could be linked with the in vivo formation of hydrogen peroxide (H_2O_2) during auto-oxidation. Other known antioxidants were tested and were found to be as effective as vitamin E in vitro, but only the tocopherols had demonstrable in vivo antioxidant activity.

In 1960, Tsen and Collier (163) attempted to determine the actual mode of action of dialuric acid on vitamin E-deficient rat RBC's, and the mechanism whereby vitamin E offers protection against hemolysis.

Using the thiobarbituric acid test[5], they demonstrated that the level of lipid peroxides does not increase until after treatment of the E-deficient RBC's with an oxidizing agent. The increase in lipid peroxides was accompanied by an increase in hemolysis. Vitamin E, when added to a suspension of deficient RBC's, significantly decreased lipid peroxide formation and subsequent hemolysis. The investigators concluded that dialuric acid catalyzes the formation of lipid peroxides in the unsaturated lipids of the RBC membrane, and that vitamin E, by virtue of its antioxidant action, inhibits peroxide formation and hemolysis.

Clinical studies on premature babies also have revealed a positive relationship between vitamin E deficiency and RBC hemolysis (58, 61, 117, 122). Lo and co-workers (98) demonstrated that a linear relationship exists between serum vitamin E levels and the degree of RBC hemolysis.

Younkin and associates (177) investigated the mechanism of hemolysis in the RBC's of vitamin E-deficient infants and found that, while there was a rapid accumulation of lipid preoxides during the 30 minutes following addition of H_2O_2, hemolysis did not occur for approximately 30 minutes.

This delay between peroxidation and hemolysis also was demonstrated by Jacob and Lux (81). They discovered that the phosphatidyl ethanolamine component of the E-deficient rat RBC membranes was destroyed after the cells were exposed to H_2O_2, but prior to hemolysis. Vitamin E added to the suspension even after exposure to the oxidizing agent, prevented both hemolysis and the loss of phosphatidyl ethanolamine. The loss of phosphatidyl ethanolamine from peroxidized vitamin E-deficient RBC membranes is illustrated in Figure 3 (81). Jacob and Lux also studied the incorporation of radioactive fatty acids into both vitamin E-deficient and sufficient oxygen-exposed RBC's. The vitamin E-deficient cells

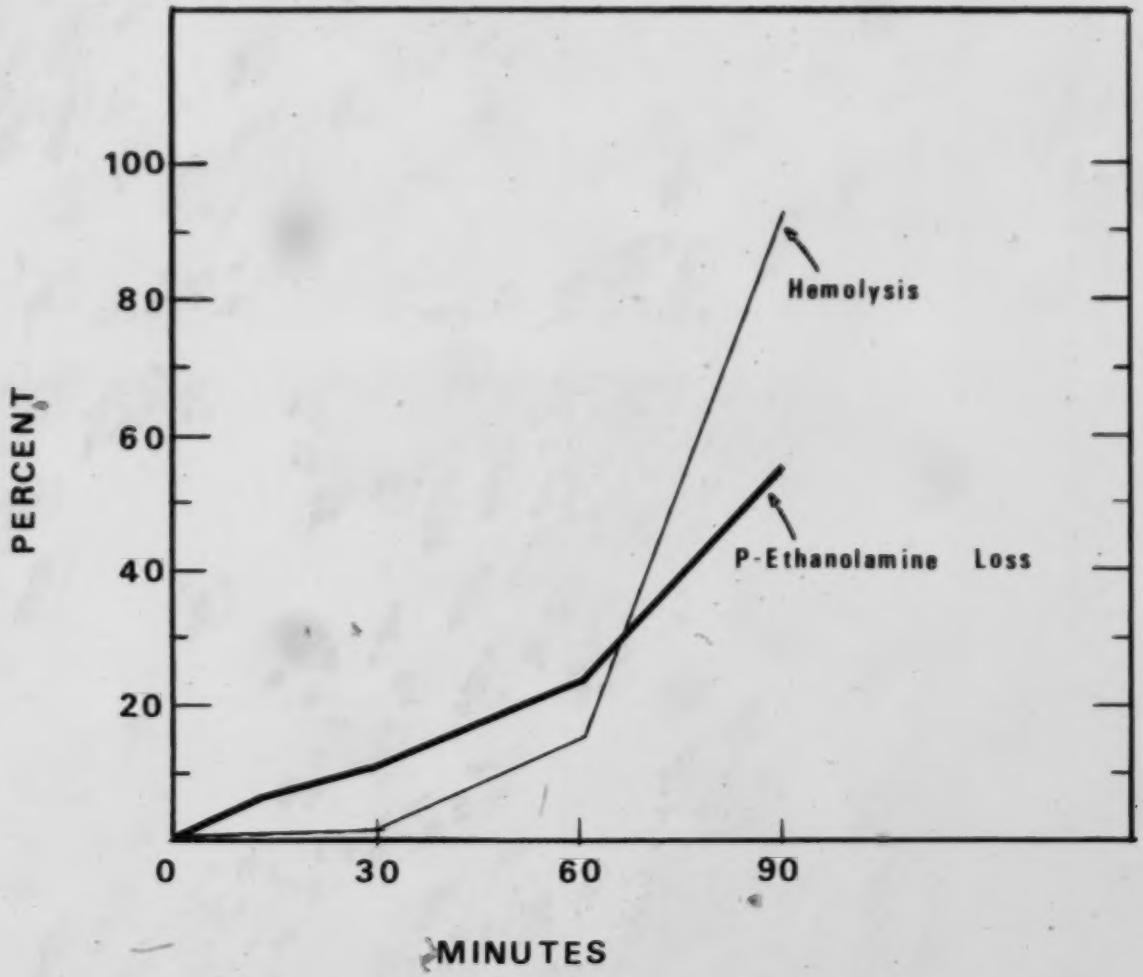


Figure 3. Sequential destruction of phosphatidyl ethanolamine and red cells by hydrogen peroxide(81).

incorporated twice the amount of labelled fatty acids into the phosphatidyl ethanolamine component as did the controls.

Heikkila and associates (74) reported that the degree of RBC hemolysis in vitamin E-deficient rats was proportional to simultaneous losses of both phosphatidyl choline, and phosphatidyl ethanolamine.

In 1975, Barker and Brin (5) reported losses of polyunsaturated fatty acids (PUFA), especially linoleic and arachidonic acids, and losses of dimethylacetyls from cleavage of plasmalogens, in dialuric acid or peroxide-treated RBC's from E-deficient rats as compared with control rats. They suggest that because phosphatidyl ethanolamine is the major plasmalogen-containing phospholipid, it is the most susceptible to attack. Barker and Brin propose that both polar and non-polar portions of membrane-bound phospholipids are partially removed from the membrane matrix during exposure of RBC's to peroxidizing reagents.

Studies by Levander et al (97) demonstrate that vitamin E status can greatly influence the response[6] of RBC's to oxidants, and to environmental toxins such as hydrazines or aminoquinolines. In similar studies, Levander et al (94-96) have studied the effect of vitamin E on the RBC filterability and morphology of non-poisoned and lead-poisoned rats. A decrease in the filterability of RBC's was observed, caused primarily by vitamin E-deficiency, but accentuated by lead poisoning. The decrease in filterability was associated with altered morphology, i.e. spherocytosis. The effect of a spherical shape to the RBC is a minimum surface to volume ratio, and an inability to stretch and bend, or squeeze through pores. The authors postulate that the spherocyte shape is an example of membrane alterations due to lipid peroxidation.

Dodge and colleagues (42), in studying the peroxide-exposed RBC's from patients with abetalipoproteinemia, suggested that hemolysis

was a consequence of lipid peroxidation. Hemolysis of the vitamin E-deficient RBC's was preceded by a decrease in PUFA, an increase in ultraviolet light absorbancy of the lipid extracts, and a decrease in both phosphatidyl ethanolamine and phosphatidyl serine.

Dillard and Tappel (39) peroxidized rat liver mitochondria and microsomes in the presence of vitamin C, and found that the production of fluorescent products was inversely proportional to the vitamin E content of the diet.

Carpenter (26) reports that in vitamin E-deficient rat testes, the levels of phospholipid PUFA are preserved, and that the level of arachidonic acid is slightly elevated. A decrease in saturated fatty acids was evident in the neutral lipid fraction. Similar results have been reported by Bieri and Andrews (9). Many investigators (9, 26, 91, 115) report a pronounced decrease in the level of docosapentaenoic acid (22:5ω6), and speculate that vitamin E deficiency inhibits the conversion of arachidonic acid to docosapentaenoic acid. However, observations on the fatty acid patterns of mitochondrial membranes from duckling liver and human epithelial cells have revealed that there also is a significant decrease in the levels of phospholipid arachidonic acid and linoleic acids as well as an increase in the levels of palmitic and oleic acids (115). Similarly, Farnsworth et al (49) report that the total PUFA content is lower in the retinal pigment epithelium of vitamin E-deficient mice than in vitamin E-supplemental mice.

A fluorescent pigment, often termed ceroid pigment, has been detected in the uteri of vitamin E-deficient rats, and in the testes and heart of vitamin E-deficient mice (38, 158, 159). Ceroid pigment in vitamin E-deficient monkeys has been observed in liver, lymph nodes, lung, spleen, retinol pigment epithelium and coronary artery cells (72).

The tissues of vitamin E-deficient monkeys who had been fed a diet based on safflower oil were more severely affected with ceroid pigment than were those of the monkeys maintained on a coconut oil diet.

D. Requirement of Vitamin E

The relationship between vitamin E deficiency and the type of dietary fat is well documented (1, 4, 23, 33, 43, 67, 79, 109, 173, 174). Ausman and Hayes (4) fed 26 juvenile monkeys diets containing 22% of the calories as either coconut or vitamin E-stripped[7] safflower oil for 32 months. By 12 months, monkeys in the unsupplemented safflower oil group developed severe anemia (hematocrit of 12-15%) depressed appetite, weight loss and very low serum vitamin E levels (less than 0.1 mg %). An in vitro hemolysis test indicated that the anemia was of a hemolytic nature. No monkeys fed coconut oil developed anemia.

Brin and co-workers (23), in studying the relationship between RBC fatty acid composition and susceptibility to vitamin E deficiency, found that feeding arachidonic acid caused a rapid onset of hemolysis and muscular dystrophy in vitamin E-depleted rabbits who had been clinically normal when maintained on stripped lard diets.

Bieri and Poukka (15) attempted to measure the amount of vitamin E required in rat RBC's to prevent in vitro hemolysis. A high peroxidizable index occurred on a corn oil diet, and an RBC vitamin E concentration of 196 μg/100 ml was found necessary to prevent significant hemolysis. On a linoleic acid-free diet (low peroxidizable index) only 122 μg/100 ml vitamin E was found necessary to prevent hemolysis.

The level of natural vitamin E present in highly unsaturated oils appears to provide adequate antioxidant protection in spite of an increased vitamin E requirement (1, 33, 79). Alfin-Slater and associates (1) fed groups of weanling male and female rats one of the following diets

for 10 weeks: cottonseed oil, corn oil, lightly hydrogenated cottonseed oil, hydrogenated soy-bean oil or hydrogenated coconut oil at 30% of the total calories of the diet. The experiment continued for 3 generations. Reproductive performance and growth for the animals on the cottonseed and corn oil diets were normal throughout all three generations. However, RBC hemolysis in the corn oil group was significantly higher than in the cottonseed oil group. Administration of vitamin E resulted in decreased hemolysis values. The investigators concluded that there is sufficient vitamin E present in both corn and cottonseed oils to satisfy in vivo vitamin E requirements (growth, reproduction, lactation) and only borderline for the peroxide hemolysis test. The authors also question the validity of the hemolysis test as an indicator of in vivo peroxidation, and suggest that there is little evidence for the in vivo antioxidant theory proposed for vitamin E.

Molenaar et al (115) also question the value of the hemolysis test as an indicator of vitamin E status, because they feel that the RBC membrane is least vulnerable to vitamin E deficiency due to the relatively high content of saturated fatty acids.

Christiansen and Wilcox (33) found no relationship between RBC hemolysis and serum vitamin E levels. They studied the RBC hemolysis and serum vitamin E levels of 10 adults on a modified PUFA diet, and 10 controls on a diet of normal fat composition for a period of 26 weeks. The serum vitamin E levels of the experimental group rose from 0.50 to 0.71 mg % whereas the vitamin E levels of the controls remained unchanged at 0.55 mg %.

Horwitt (79) and Witting (173, 174) in discussing the human requirement for vitamin E, emphasize that an individuals vitamin E: PUFA status is determined by the average intake over a period of months, or

even years. The fatty acid composition of human tissue eventually will reflect the fatty acid composition of the diet if the diet is fed for a sufficient period of time. This is because the one-half turnover rate of linoleic acid is approximately 26 mos (174). If an individual, who previously had consumed a high PUFA diet for several years, changed his diet to one composed chiefly of animal fats, the tocopherol requirement would remain elevated for 2 to 3 years, due to the high linoleate content of the body tissue.

Horwitt (79) has suggested that, in calculating the vitamin E requirement of an individual, the percentage of PUFA in the total dietary lipids, as well as the actual number of grams of PUFA both be taken into account, because the time taken to reach tissue equilibrium is affected by the amount of total dietary fat.

E. Postulated Role of Vitamin E: Importance in Membrane Integrity

To account for the evidence that high dietary levels of PUFA increase the requirement for vitamin E, a different theory as to the role of vitamin E in membrane permeability and stability was suggested by Lucy (102) in 1972. He maintains that the antioxidant hypothesis has never been proven in vivo, and proposes that vitamin E plays a role in membranes that is comparable to the role of cholesterol. The difference is that vitamin E is more interactive with unsaturated lipid residues, particularly arachidonic acid. In this hypothesis, membranes with a high level of PUFA necessarily will require high levels of vitamin E for structural stability. With an absence of vitamin E, it is hypothesized that the presence of unsaturated fatty acids will increase the accessibility of membrane-bound phospholipids to membrane-bound phospholipases.

Pappu and associates (124) found that there was a more than two-fold increase in phospholipase A_2 activity of liver mitochondria isolated from vitamin E-deficient rats compared with that in normal rats.

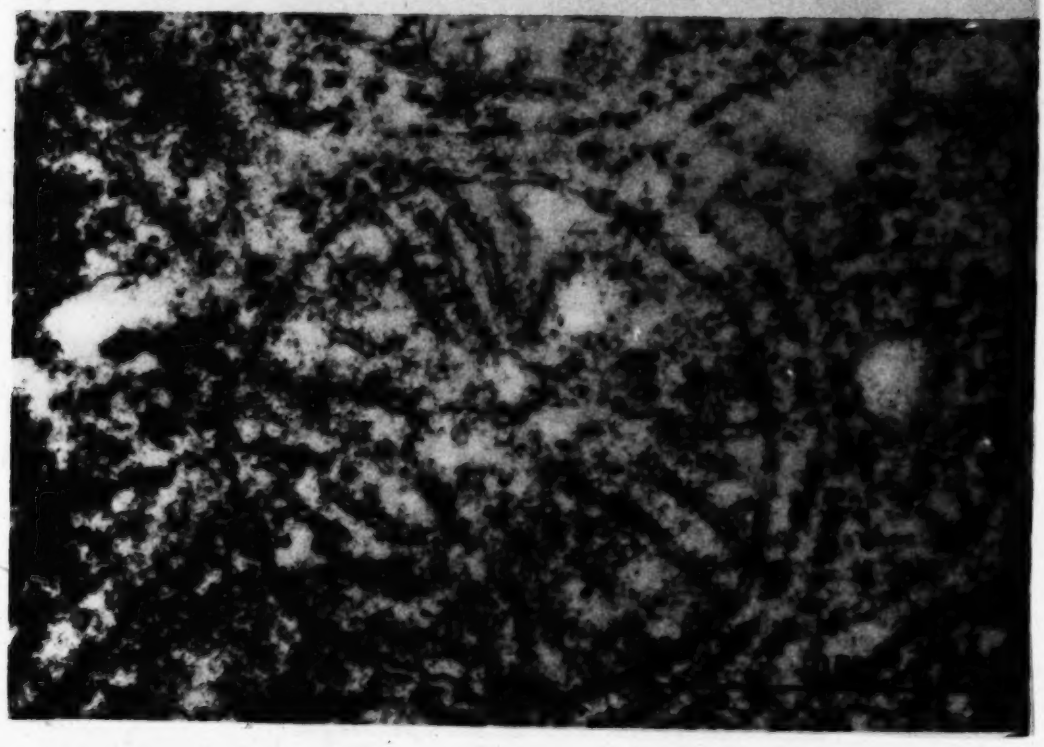
Hulstaert and co-workers (80) studied the effect of vitamin E deficiency on cellular membranes and membrane-bound enzymes. The livers of vitamin E-deficient and control Pekin ducklings were examined histochemically, chytochemically and biochemically. There was a 30% increase in cell volume associated with vitamin E deficiency, leading to an increase of protein, phospholipid and RNA per cell. An increase in the activity of 5' nucleotidase (plasma membrane) and glucose-6-phosphatase (endoplasmic reticulum) was demonstrated biochemically. The thickness of the outer mitochondrial membrane was found to be smaller in vitamin E deficiency, whereas the thickness of the endoplasmic reticulum was greater. Other investigators (55, 73) have observed that vitamin E has an effect on the ultrastructure of subcellular membranes (see Discussion).

Molenaar and colleages (115) have studied intensively the effect of vitamin E deficiency on the ultrastructure and chemical composition of the jejunal and liver epithelial cells of vitamin E-deficient and control Pekin ducklings, and of 2 children with abetalipoproteinemia. The membranes of jejunal epithelial cells could not be visualized by electron microscopy, and negative staining with osmium tetroxide, which binds to the double bonds of unsaturated fatty acids, produced poor pictures due to a decrease in membrane contrast compared with control films. The 2 patients were treated with vitamin E and a normal cellular ultrastructure appeared after 4 months of therapy. The loss of contrast associated with vitamin E deficiency appeared first and was most marked in the outer mitochondrial membrane (see Plate 1), followed by the endoplasmic

Plate 1 Mitochondria from jejunal epithelial cells of vitamin E-deficient and control Pekin ducklings.

(a) Jejunal epithelial cell from a vitamin E-deficient duckling. There is a big difference in contrast between the inner and outer mitochondrial membranes.

(b) Jejunal epithelial cell from a normal duckling. The electron density of the mitochondrial membrane is similar. X140,000 (reprinted from reference 115).



reticular and nuclear outer membrane. The microsomal membranes did not differ from controls.

Grinna (60) has reported that liver microsomal and mitochondrial membrane vitamin E concentrations reflect the total liver vitamin E content. No significant difference was found between the total phospholipid/protein ratio of the microsomal or mitochondrial fractions of vitamin E-deficient and supplemented rats (see Table 1), although the amount of phospholipid in the vitamin E-deficient mitochondrial fraction did appear to be slightly decreased. This observation is discussed further in the Discussion. Grinna also reported that there was no correlation between dietary vitamin E and lipid peroxidation[9] in either the microsomal or mitochondrial membrane fraction. Grinna questions the value of measuring pigment fluorescence to determine antioxidant status. When the extent of fluorescence of microsomal and mitochondrial membranes was investigated with an ANS probe[10] there was a significant decrease of fluorescence observed in the mitochondrial fraction which correlated well with tocopherol status (see Table 1).

Many changes at the subcellular level indicate a requirement of biological membranes (the phospholipid bilayer) for vitamin E. Studies have demonstrated that vitamin E affects mitochondrial membrane permeability to phosphate (77) and RBC membrane resistance to the toxic effects of lead and other environmental agents (94-97).

Since the membrane model of Singer and Nicolson describes the membrane as a "two-dimensionally oriented solution of globular proteins in a viscous phospholipid bilayer solvent[11]", one would expect vitamin E deficiency to have an effect on membrane proteins.

Studies by various investigators reveal that vitamin E deficiency may be associated with alterations in the levels of specific pro-

Table 1. Effect of dietary vitamin E on rat liver membrane phospholipid, pigment fluorescence and ANS fluorescence(60).

	Microsomes		Mitochondria		
	C[a]	E[b]	C	E	
Alpha-tocopherol[c]	0.91	-[d]	6.5	-	
Phospholipid[e]	0.37	0.38	0.19	0.15	NS[h]
Pigment fluorescence[f]	0.32	0.42	0.32	0.30	NS
ANS fluorescence[g]	6.6	6.4	17.3	13.5	*[i]

a. Indicates control group.

b. Indicates experimental group.

c. Values are expressed in μg membrane alpha-tocopherol per 100 mg membrane protein.

d. Indicates not detectable.

e. Values are expressed as mg total membrane phospholipid per mg membrane protein.

f. Values are expressed in μamps/mg membrane protein.

g. Values are expressed in μamps/mg membrane protein.

h. NS indicates no statistical significance between groups.

i. * Indicates a statistical difference ($p < .10$) between the mean of both groups.

teins and enzymes (27, 30, 40, 71, 80, 114, 116, 120, 124, 133, 155, 156) while some investigators have discovered alterations in cellular energy metabolism in association with vitamin E deficiency (25, 28, 73, 144, 178).

Swierczynski et al (155) have demonstrated that vitamin E stimulates the ATPase activity of intact mitochondria. Santiago et al (141) and Segovia et al (145) have shown that antioxidants such as BHT[12] have a protective effect against mitochondrial phospholipid degredation and losses of mitochondrial ATPase active, and that losses of mitochondrial ATPase activity can be correlated with peroxidation of cardiolipin induced by ascorbic acid.

Many of the biochemical abnormalities associated with vitamin E deficiency reflect defects at the subcellular level which explains the wide range of deficiency signs. For example, rabbit muscle phopholipid, which has a higher content of arachidonic acid relative to linoleic acid, is the tissue most susceptible to vitamin E deficiency (muscular dystrophy), whereas the rabbit RBC, which has more linoleic acid relative to arachidonic acid, is immune from the effects of vitamin E deficiency. This is opposite to what occurs in the rat (15, 23, 73, 91, 120, 158). Vitamin E protects the RBC membrane from in vivo hemolysis by stablizing the phospholipid bilayer in some manner. Perhaps the function of vitamin E eventually will be found to have two components, the first as an antioxidant, the second as a physical stabilizer of membranes of particular relevance to this research is the evidence that RBC hemolysis is associated with a loss of certain phospholipids. Also, reported changes in the ultrastructure and biochemistry of subcellular organelles suggest that vitamin E deficiency may affect their phospholipid composition in a manner similar to that suggested for the RBC membrane. The phospholipids of subcellular

membranes and especially those of mitochondria, are known to contain a large proportion[13] of arachidonic acid (34), and therefore should be particularly susceptible to peroxidation.

FOOTNOTES

1. The terms vitamin E and tocopherol are commonly used interchangeably, as they are in this paper. However, the term vitamin E, which is more commonly used, will be employed more often.
2. Cardiac muscle is particularly sensitive to low regimens (78).
3. Two cases of human cerebellar encephalomalacia related to tocopherol deficiency have been reported.
4. Storage occurs in the vacuoles of fat cells and in cell membranes, the latter constituting an extremely large surface area (78, 79, 115).
5. The TBA test measures the oxidation products of linoleic and linolenic acid.
6. Indicated by the filterability of erythrocytes.
7. Half the monkeys in the stripped safflower oil group received vitamin E.
8. No information is given on the exact number of rats used in the experiment. The data is presented as a mean of 5 independent experiments, each involving groups of rats.
9. Production of pigment fluorescene as measured by the TBA test.
10. The ANS molecule binds to hydrophobic regions of membranes and fluorescence increased where the probe is a hydrophobic environment i.e. more lipid.
11. Quoted in reference 115.
12. butylated hydroxytoluence.
13. Approximately 50% arachidonic acid.

III. EXPERIMENTAL PROCEDURE

A. Purpose of Experiment

The purpose of this study was to determine whether the levels of 3 subcellular membrane phospholipids, phosphatidyl choline (PC), phosphatidyl ethanolamine (PE) and diphosphatidyl glycerol[1] (DPG), are reduced in the liver and testes tissue[2] of vitamin E-deficient rats. Determination of serum and tissue (liver and testes) alpha-tocopherol levels were the objective parameters used to measure vitamin E status. A subjective evaluation of the animals at sacrifice also was undertaken, as well as a subjective assessment of the subcellular organelles using negative staining electron microscopy techniques. A two-dimensional thin-layer chromatographic method was utilized to separate the 3 phospholipids, and phosphorus levels were measured to quantitate the individual phospholipids.

B. Subjects

Forty 50 g weanling (21 day old) male rats were ordered from a closed colony Sprague-Dawley strain to comprise the subjects for the investigation. Upon arrival at the laboratory it was apparent that the animals were approximately 50% heavier and 5 to 6 days older than was anticipated. The animals immediately were allocated to either the control (vitamin E-supplemented) or experimental (vitamin E-deficient) groups on the basis of weight, age and litter distribution. The general characteristics of the control and experimental rats at the onset of the feeding trial are displayed in Table 2. The animals ranged in age from 25 to 29 days. The mean age for the experimental group was 26 days with a mean age of 27 days for the control group. Initial weights for the experimen-

Table 2. General characteristics of control and experimental groups at onset of feeding trial.

Rat Number	Weight[a]		Age[b]		Litter Group	
	C[c]	E[d]	C	E	C	E
1	63.0	65.0	25	25	1	1
2	67.0	67.0	25	25	1	1
3	71.0	70.0	26	26	2	2
4	72.0	70.0	26	26	2	2
5	73.0	73.0	26	26	2	2
6	75.0	73.0	26	26	3	3
7	78.0	75.0	26	26	3	3
8	78.0	77.0	26	26	3	3
9	79.0	79.0	26	26	4	4
10	80.0	83.0	27	26	5	4
11	81.0	84.0	27	26	5	4
12	83.0	84.0	27	26	5	4
13	83.0	85.0	27	26	5	4
14	84.0	86.0	27	27	5	5
15	85.0	88.0	27	27	6	5
16	88.0	88.0	27	27	6	6
17	90.0	96.0	28	27	7	6
18	91.0	97.0	28	27	7	6
19	96.0	98.0	28	28	7	7
20	98.0	105.0	29	28	8	7
MEAN[e]	81	82 (NS)[g]	27	26 (NS)		
SEM[f]	9.2	11.1	1.0	0.8		

a. Values expressed to nearest g.

b. Values expressed in days since birth.

c. Indicates control group.

d. Indicates experimental group.

e. Mean value expressed to nearest g.

f. Standard error of the mean.

g. NS indicates no statistical difference between the mean of both groups.

tal group ranged from 65 g to 105 g with a mean of 82 g. The control group had a similar range, from 63 to 98 g, with a mean of 81 g. The small differences in the mean age and weight of the control versus the experimental groups were not significant.

The animals were housed in individual, identical plastic cages (25 cm x 20 cm x 48 cm), fitted with wire mesh lids, 25 ml water bottles, tag holders and metabolic feeding cups. Bedding consisted of sand and wood chip absorbant. The cages were all kept in the same room under constant ideal temperature, humidity and lighting conditions.

C. Diet

The 20 rats in the experimental group were placed on a powdered vitamin E-deficient diet.[3] The 20 rats in the control group received the same diet mixture, but these animals were supplemented with 250 mg of dl-alpha-tocopherol acetate per kg of powdered food.[4] The level of vitamin E supplementation selected was within the range of amounts chosen by other researchers (5, 9, 49, 60, 71, 103, 124), and was greater than the level recommended for the rat (135), yet well below what could be considered a megavitamin dosage.

The diet mixture used (see Table 3) was based on the formulation of Draper et al (44). The most important aspect of the Draper diet is the use of 10% of the diet as stripped corn oil, which provides 20% of the total calories high in PUFA. The salt and vitamin diet fortification mixtures (see Appendicies I and II) contained all minerals and vitamins known to be essential for rat nutrition, with the exception of vitamin E.

The powdered diet formulation was selected because it would be more manageable to weigh, and mixing with the vitamin E would be considerably easier, and would result in a more homogeneous blend than if the pellet form were used. In order to maintain standardization of the

Table 3. Vitamin E-deficient rat diet of Draper et al.

Ingredient	% Total Weight	% Total Calories
Corn oil-tocopherol stripped	10.0	20
Glucose	65.4	60
Salt mixture 4164[a]	4.0	-
Vitamin Free Casein	20.0	20
Vitamin Diet Fortification[b] Mixture except vitamin E	0.6	-

a. See Appendix I.

b. See Appendix II.

control diet, a standardized procedure (see Appendix III) for mixing vitamin E into the vitamin E-deficient powdered diet was carried out at all times. In order to prevent rancidity and bacterial contamination the food was held at -20°C. Weekly portions of the 2 mixtures were removed from the freezer and held in the refrigerator at 5°C.

Control and experimental groups were pair-fed for the first 3 weeks, and subsequently were allowed to feed ad libitum. Ad libitum feeding was allowed because it was apparent that the food intake and weight gain of the 2 groups were similar, and not related to the pair-controlled feeding. Regular City of Edmonton water, which was noted to be uncontamined with selenium[5] (see Appendix IV), also was fed ad libitum to all rats.

A record of daily food intake and weight gain was kept of each rat during the initial 21 days of the feeding trial, when pair-feeding was being employed. During the remaining 24 to 28 wks of the feeding trial, records were maintained on a weekly basis.

D. Collection of Samples

Two rats from each group were selected randomly and sacrificed at 3 to 4 day intervals, beginning at the 24th and finishing at the end of the 28th week of the feeding trial. The rats were fasted overnight to deplete liver glycogen stores (24, 57, 63, 76, 126, 139, 142). To avoid any discrepancies of results due to possible effects of circadian periodocity, all rats were sacrificed between 9:30 and 11:00 a.m. Before sacrifice, the final weight of each rat was recorded in order to complete the growth/weight chart.

Each animal was anaesthetized with ether and examined carefully for evidence of nasal porphyrin secretion, greasy-rough coat, or skeletal deformity. Then the abdomen was opened and blood was withdrawn from the heart through the opened diaphragm using a 10 ml plastic syringe fitted

with a size 20 neddle. The blood was transferred immediately from the syringe to a labelled 15 ml glass test tube, sealed with parafilm, and kept on ice. Two microhematocrit tubes were 2/3 filled with blood pooled in the mediastinum around the heart. The end which was not wet with blood was plugged with modelling clay and the tubes were placed into a labelled area of a hematocrit rack.

The liver and testes were removed and placed into a labelled 100 ml pyrex beaker filled with ice-cold isotonic KCL. The ice-cold state was maintained by placing all beakers in a bucket of ice.

Following collection of all the required tissue samples, the hematocrit samples were read immediately using the procedures outlined in Appendix V.

E. Storage of Tissue

The liver tissue was blotted dry and weighed. The right lobes of both control rats were pooled, and 1 g samples of the liver tissue were prepared for subcellular membrane (SM) pellet isolation[6] as outlined in Appendix VI. The remaining liver tissue was placed into a labelled 10 ml pyrex vial. The vial was sealed under nitrogen, capped tightly, covered with parafilm, wrapped in aluminum foil and relabelled. This procedure was repeated for the experimental group. The pair of rat testes were weighed for each rat and the weights recorded. One testis from each control rat was selected, and the pooled tissue was prepared for SM pellet isolation as outlined in Appendix VI. The remaining testis from each control rat was placed into a labelled 10 ml pyrex vial and treated in an identical manner as the liver tissue. The testes from the experimental group were treated exactly as the controls. All vials (liver and testis, control and experimental) were then placed into a 600 ml size self-sealing plastic bag and again sealed under nitrogen. The bag was placed into a

freezer and stored at -60°C.

Approximately 1 hr after collection, the clotted blood samples were centrifuged at 1,200 rpm for 10 min. This resulted in the separation of blood serum from the cellular components of blood. Using a clean Pasteur pipette with each sample, the serum (top layer) was pipetted from the tubes and placed into labelled plastic 5 ml tubes. The tubes were sealed tightly under nitrogen, covered with plastic caps, wrapped in parafilm and then in aluminum foil and labelled again. The tubes were wrapped with aluminum foil to prevent the vitamin E from being oxidized by light (11, 13, 14, 21, 46, 52, 78, 86, 100, 130, 131).

Two months after the last group of animals was sacrificed, the frozen serum samples were thawed, and subsequently were analyzed for serum vitamin E and carotene as outlined in Appendix VII. Since vitamin E is a light sensitive vitamin, care was taken to carry out all analytical procedures in a dimly lighted room. All working standards and reagents were stored in brown bottles, and care always was taken to seal the bottles with nitrogen in order to eliminate as much atmospheric oxygen as possible. One hundred percent distilled alcohol (ethanol) was obtained from the Alberta Liquor Control Board and used at all times for the extraction procedure whenever reagent grade 100% alcohol was required. The laboratory grade ethanol was found to be only 98% pure.

An unpaired t-test was used to test the significance of difference between the serum vitamin E values of the experimental group and those of the control group. The unpaired t-test also was applied to test the significance of difference between the hematocrit readings, final weights and tissue (liver and testes) weights of the control group versus the vitamin E-deficient group.

The paired t-test was not used for statistical analysis even

though the rats were paired for the initial month of the feeding trial. Paired comparison is frequently used when either the same subject is tested twice, or when the 2 subjects to be compared share the same environmental experience. Paired comparisons must be planned for and not performed after the data has been attained (113). While the standard t-test often neglects to account for the variance component among individuals, it was chosen as the statistical test because the rats, unlike most human experimental subjects, were from a genetically inbred species. When the subject-to-subject variability is eliminated, the paired t-test provides less information for testing the difference between 2 population means because the number of degrees of freedom for the t-test statistic is reduced by half, thus it is more difficult to detect a difference in means (150).

At the time of sacrifice, the presence or absence of nasal porphyria, greasy-rough coat and skeletal deformity was noted as either present (+) or absent (-). No attempt was made to quantify such results. To determine whether any significant differences existed between the control and experimental groups, it was necessary to use a non-parametric statistical test (113, 150). The sign test was used to test the hypothesis that the distribution of scores was identical.

F. Preparation and Storage of Phospholipid and Protein Samples

Preparation of the SM pellet as outlined in Appendix VI was initiated within 30 min of sacrificing the animal. The weight of the SM pellet was calculated by subtracting the weight of the centrifuge tube before pellet isolation from the weight of the tube after isolation. Often, water drops would remain in the tube after the final decantation step, thus care was taken to blot out (using a long cotton swab) any excess water that would give a falsely high reading to the pellet weight. Approx-

imately 2 ml distilled water then was added to the pellet and the resulting suspension mixed for one minute of a vortex mixer. The suspension was poured carefully into a 10 ml graduated centrifuge tube and distilled water was added up to a volume of 4 mls.

One milliliter of the pellet suspension was pipetted into a 5 ml plastic tube, tightly stoppered, sealed with tape and stored at -20°C for subsequent Lowry protein determinations. One drop of the mixture was withdrawn using a Pasteur pipette, and samples were prepared for electron microscope evaluation using the negative staining technique outlined in Appendix VIII. This procedure was carried out to subjectively assess the type and hemogenicity of SM isolated, and to enable subjective comparison of the experimental membranes with the control membranes.

The remaining 3 ml of pellet suspension were poured into a 25 ml glass round-bottomed centrifuge tube and a procedure was followed for phospholipid extraction as outlined in Appendix IX. The phospholipid extracts were resuspended to 1.0 ml in chloroform:methanol (2:1)[7] and were pipetted into labelled vials and sealed under nitrogen. The caps were wrapped with tape, a layer of parafilm[8] and finally, aluminum foil. After being relabelled they were placed into a special box which had been fitted with cardboard rows to prevent the vials from tipping over during freezer storage. The lipid extracts were maintained at -60°C for 8 mos.

G. Subcellular Membrane Pellet Protein

After 4 mos of storage at -60°C, the 1 ml frozen suspensions of SM in distilled water were thawed and analyzed for SM protein levels. Triplicate aliquots from dilutions of each sample were prepared and analyzed using the Lowry protein method (see Appendix X). The results were calculated and the values of the 2 groups were compared using the un-

paired t-test.

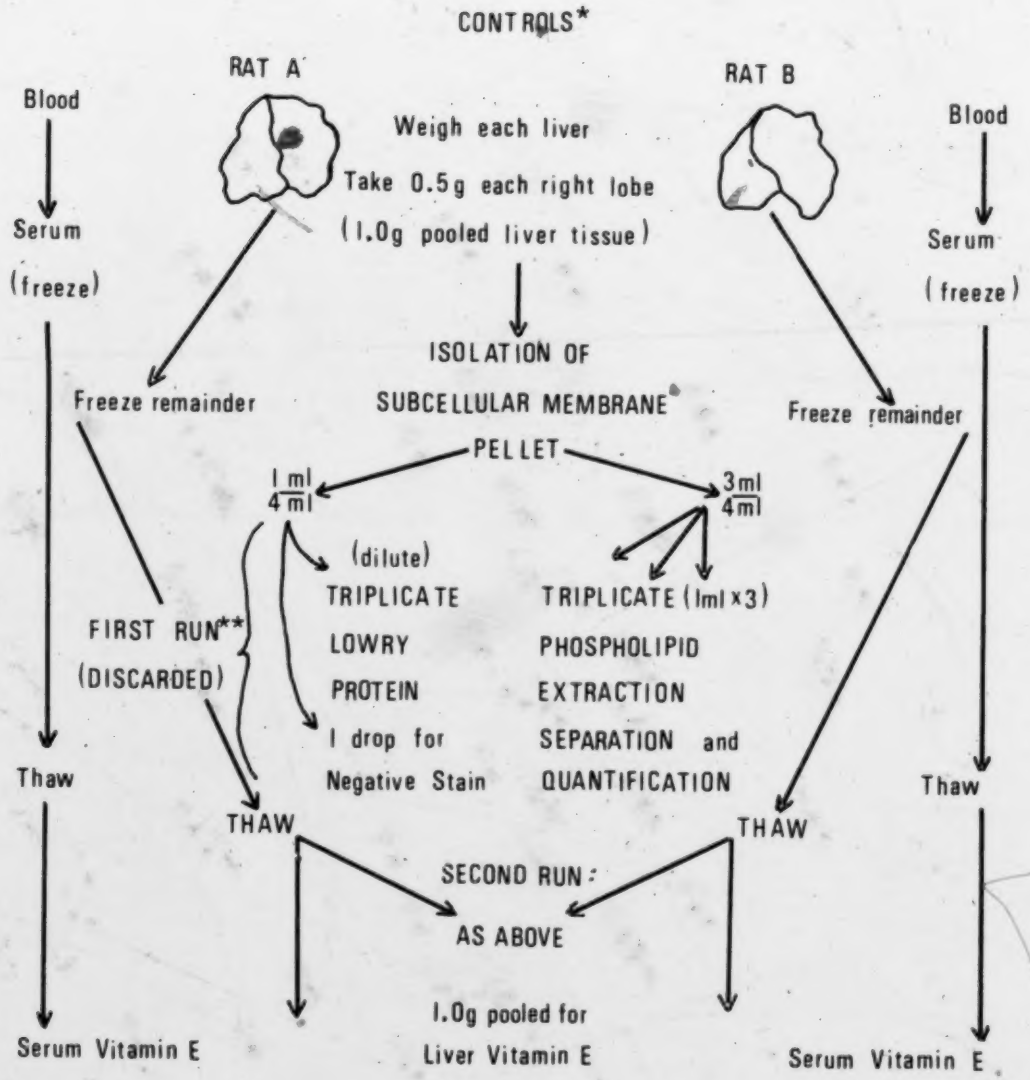
H. Thin Layer Chromatography

The preparation and procedure for separating the 3 SM phospholipids was followed as outlined in Appendix XI. After several analyses were performed on a variety of the frozen lipid extracts, it was obvious that the DPG component had decomposed to such an extent that it could not be successfully separated out and quantitated. The frozen lipid extracts were completely discarded.

The pellet isolation, protein determination, phospholipid extraction and chromatography procedures were all repeated once more using the tissue that, 8 mos previously, had been frozen for tissue vitamin E anaylses. Figures 4 and 5 portray the various treatments performed on all the tissue analyzed in the study. It will be noted that, while sufficient tissue was present to permit both the successful isolation of SM pellets for all samples and the refreezing of tissue for subsequent tissue vitamin E analyses, the testes tissue had to be pooled differently. For the second run, 1 testes from a rat was used for the phospholipid analyses, and 1 testes (other rat) was used for subsequent vitamin E analysis. Also, modifications were made in the extraction procedures (see Appendices VI and IX) to accommodate the reduction in tissue weight. With that exception, the experimental procedure continued as previously outlined. When the tissue homogenization, pellet isolation and lipid extraction steps were repeated, the 3 procedures were carried out as a continuum, and the phospholipids were separated using thin layer chromatography within 48 hrs of the initial tissue homogenization procedure. The negative staining technique outlined in Appendix VIII again was used as a means for subjectively assessing the appearance of SM isolated.

The levels of SM phospholipids from the frozen liver and testes

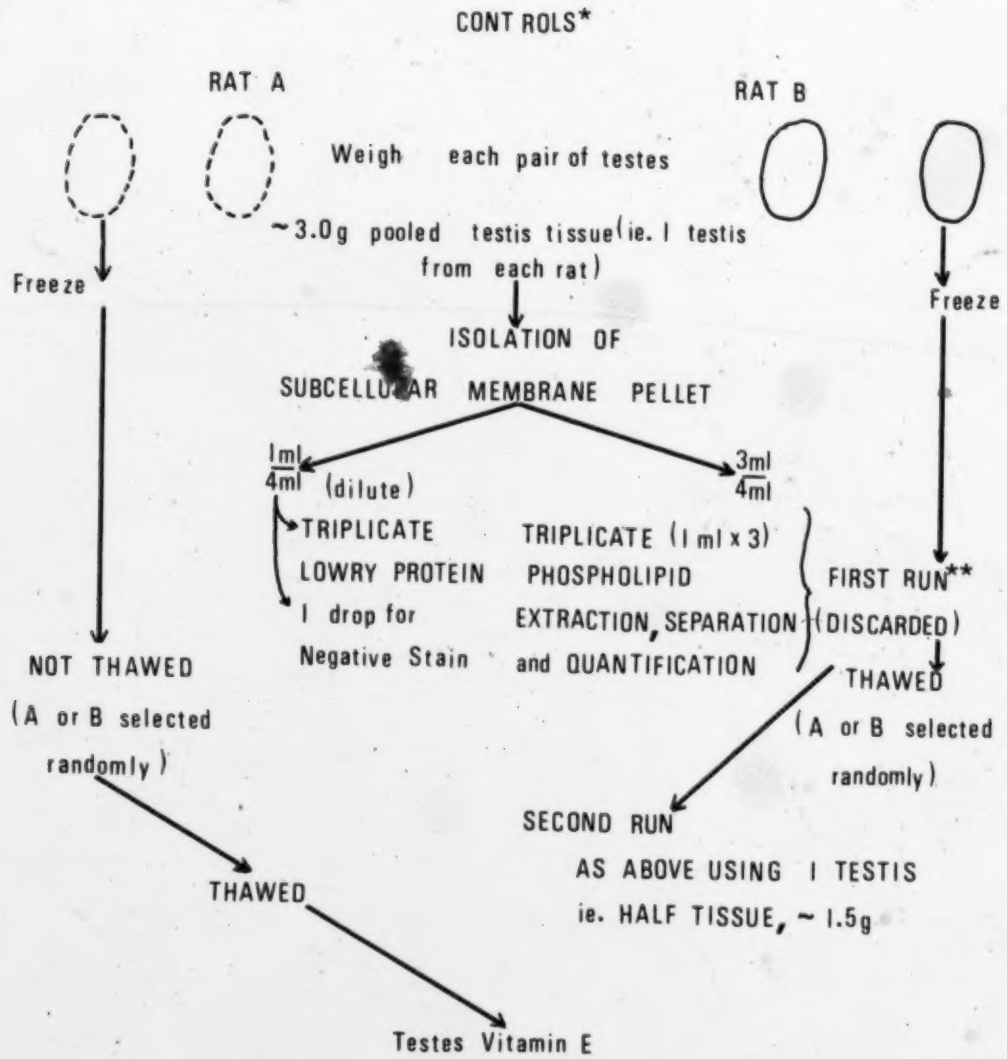
Figure 4. Flow diagram of treatments performed on liver tissue and blood from control rats used in this study.



* These treatments were repeated exactly for all experimental animals used in this study.

** First and second run refer to changes in the methodology (see Appendices VI and IX) with respect to extraction volume.

Figure 5. Flow diagram of treatments performed on testis tissue from control rats used in this study.



* These treatments were repeated exactly for all experimental animals used in this study.

** First and second run refer to changes in the methodology (see Appendices VI and IX) with respect to extraction volumes.

tissue of a control rat were compared with the levels of SM phospholipids from the liver and testes of a freshly killed, 6 mo old male rat, who had been maintained on a standard 4% fat laboratory rat chow diet. This step was undertaken to determine whether any demonstrated loss of phospholipids, resulting from the tissue being frozen for eight months, would be of a magnitude to question proceeding with the experimental analysis. The findings are outlined in Appendix XII, and are discussed in the Discussion.

I. Phosphorus Determinations

Phosphorus determinations were carried out on the day following the chromatographic separation of the 3 phospholipids. The separate spots were completely removed[9] into labelled 10 ml pyrex test tubes, and analyzed for phosphorus content, based on a modification of the method by Bartlett (see Appendix XII). The unpaired t-test was used to test the significance of difference between the individual phospholipid levels of the control versus the experimental group.

J. Tissue Alpha-Tocopherol

The tissue alpha-tocopherol determinations were the final analyses, and were carried out 9 mos after the last group of animals was sacrificed, ie. 7 mos after the serum vitamin E determinations. For the tissue alpha-tocopherol determinations similar precautions as those for serum vitamin E were undertaken with respect to the light sensitive behavior of vitamin E. The laboratory work was conducted over 4 consecutive 2-day periods. On the first day of each run, the tissue lipid was extracted according to a modification of Folch's method (see Appendix IX). The samples were placed in 15 ml pyrex tubes fitted with a screw cap, sealed under nitrogen, and covered with aluminum foil before being stored overnight in the freezer at -60°C. On the following day the

samples were analyzed for tissue alpha-tocopherol levels, according to a modification of Quaife's method (see Appendix VII). The resulting tissue alpha-tocopherol and beta-carotene values of the control and experimental groups were compared using the unpaired t-test.

FOOTNOTES

1. Also termed cardiolipin.

2. Since it was not practical, or felt to be necessary, to study all rat tissues, it was decided to select 2 tissues. Testis tissue was selected because of its' known sensitivity to vitamin E (9, 12, 26, 121, 178) and liver tissue was selected because it is a commonly investigated tissue due to its' importance in metabolism (25, 27, 28, 40, 55, 71, 124).

3. Nutritional Biochemicals of Ohio.

4. Equivalent to 5 mg vitamin E per day at 20 g food per day.

5. Since selenium has been demonstrated to act as an antioxidant and partially replace the function of vitamin E (22, 40, 49, 64, 90, 146, 167) it was considered important to check that selenium was not present in the water supply.

6. The initial objective of the study was to investigate the phospholipid composition of mitochondria membranes, and the laboratory method used was one for isolation of mitochondria. However, since sophisticated steps were not undertaken to objectively measure the purity of the isolated mitochondrial fraction (such as the use of marker enzymes), the term subcellular membranes (SM) is used instead throughout the report. The negative staining procedure employed is recognized as a useful tool in the subjective evaluation of membrane purity and is discussed further in the Discussion section.

7. To prevent oxidation of the lipids, BHT (1 g/l) was added to all chloroform;Methanol used in this experiment.

8. Because parafilm is a petroleum byproduct, and soluble in organic solvents, care was taken to not put parafilm directly over vials containing chloroform-methanol for storage. Instead, the caps were sealed with cork and were first wrapped with tape.

9. The same surface area (4.0 cm^2) of silica gel for each of the 3 phospholipids, both for standards and unknowns, was scraped from the chromatographic plates.

IV. DISCUSSION OF METHODOLOGY

Alpha-Tocopherol

The main chemical feature of tocopherols is the susceptibility of their hydroxyl group to oxidation. This oxidation proceeds rapidly in air or when certain metal ions, such as iron, are present in solution (13). The basis for the Emmerie-Engel procedure for determination of tocopherol status is a reaction of the sample with ferric chloride ($FeCl_3$) and alpha, alpha^1-dipyridyl to produce a red color. The intensity of color development (oxidation) in pure solutions of vitamin E is directly proportional to the amount of tocopherol present. However, the Emmerie-Engel reaction is not specific for tocopherols, since lipid extracts of biological specimens show an increase in color over time, when compared with standard vitamin E solutions. The reaction is measuring extraneous reducing materials in addition to tocopherols (130). However, according to Bieri (13) simple spectrophotometric methods are fine for most laboratory purposes, because there is no evidence that serum lipids other than carotenoids are present in amounts sufficient enough to interfere with the Emmerie-Engel reaction.

Quaife and Harris (130) reported on a macro method involving both lipid extraction and a mild hydrogenation process, because alcoholic solutions of carotene were found to have no effect on Emmerie-Engel reagents after hydrogenation (130). Subsequently, these investigators eliminated the hydrogenation step because it was difficult to avoid significant loss of tocopherol when using microvolumes of serum or plasma. Instead, light absorption due to carotenoids alone was measured at 455 mμ, and a correction was made (see Discussion) for the contribution of caro-

tene to the Emmerie-Engel reaction (131). Similar methods have been reported by other researchers (9, 70, 88). Bieri found that the values obtained by the spectrophotometric method were slightly higher than those obtained using chromatography (9).

Two investigators recommend that to ensure total liberation of vitamin E, a saponification step be introduced prior to the extraction procedure (86, 88). During saponification the plasma/serum proteins are precipitated by ethyl alcohol in the presence of pyrogallol. Pyrogallol is added to prevent extensive loss of vitamin E which occurs during saponification. The amount of pyrogallol required to prevent oxidation of vitamin E is quite large (180 mg per ml) and some investigators feel that these large amounts may interfere with the final tocopherol estimation (13, 164). Bieri (13) has reported that when more than 75 mg of pyrogallol per ml is used black oxidation products (of pyrogallol) cause streaking on thin layer chromatography plates.

Other methods for determining vitamin E status have recently been developed, including spectrophotofluorometric methods (46, 66), thin-layer chromatographic separation of individual tocopherols (11, 13, 100) and gas-liquid separation of individual tocopherols (14, 92, 100).

Since the purpose of vitamin E measurement in this study was to demonstrate a relative absence (deficiency state) or presence (control) of vitamin E between the 2 groups, a simple spectrophotometric method was chosen. The isolation and quantification of individual tocopherols by chromatographic means was felt to be not necessary since this was not the primary purpose of the study. It was decided not to select a method involving either hydrogenation or saponification, but rather to measure the actual level of carotenoids and to correct for their interference. Good standard curves and results were achieved using alpha, alpha[1]-

dipyridyl and ferric chloride as reagents, and with some practice, the need for timed reactions proved no great difficulty. Thus, the spectrophotometric micro-method of Quaife et al (131) was selected as the method for serum and tissue vitamin E determinations.

Subcellular Membrane Isolation and Identification

The isolation of SM components is a lengthy process, complicated by the fact that the initial homogenization of tissue involves cell disruption. In order to prevent heating of the tissue during homogenization, most methods suggest that the disruption process be carried out with the tubes immersed in ice-water. Depending on the cell fraction desired, either the pellet or the supernatant from the initial centrifugation is collected, and further centrifugations are carried out at varying centrifugal forces on supernatant or pellet fractions. The overall process is greatly lengthened if repeated washings are done on the final pellet (63).

The question always arises as to whether or not the differential centrifugation process has produced artifacts. These include both the morphological alteration of subcellular components and the redistribution of biochemical properties (76). A variety of methods (biochemical, negative-stains, thin-layer sections) can be used to identify the cellular components under study (63, 76, 142, 151, 160).

According to Hogeboom (76), when mitochondrial preparations are isolated in water they quickly lose morphological integrity, appearing larger and paler as a result of swelling. While using isotonic saline (or other salts such as KCl or K_2SO_4) often causes the mitochondria to agglutinate in small clumps, they are better preserved. However, they still do not appear morphologically intact. When isotonic sucrose (ie. 0.25 M sucrose) is used, the mitochondria appear morphologically identi-

cal to those prepared in isotonic saline, but there is less clumping. Hogeboom prefers a hypertonic solution (0.88 M) of sucrose because the mitochondrial formation appears more rod-like than spherical. Schneider (142) also reports extensive agglutination of mitochondria when isotonic saline is used in the centrifugation process. He recommends using 0.25 M sucrose and 4 to 5 final sedimentations to increase the purity of the preparation. The nitrogen content of the mitochondria was found to decrease 7% from the first to the second sedimentation, but further centrifugations did not appreciably alter the mitochondrial protein level. Octanoxidase activity remained constant after 4 sedimentations.

Sulimovici (151) measured the glucose-6-phosphatase activity of rat testes mitochondrial preparations in order to establish mitochondrial purity. The relative presence of glucose-6-phosphatase, a microsomal marker enzyme, was assumed to reflect microsomal contamination. After 5 successive washings with 0.154 M KCl glucose-6-phosphatase activity was reduced to 1.66 nmoles/mg protein from 36.88 nmoles/mg protein. This point emphasizes the importance of repeated washings (as outlined by Schneider) to increase sample purity as well as the application of biochemical means to establish the purity. Negative stain preparations of the sample revealed that the bulk of the ribosomes and rough endoplasmic reticulum were removed after 3 successive washings with 0.154 M KCl. The circular shape of the mitochondria was attributed to sonication of the sample prior to the extraction procedure.

The method of Schneider was used for the isolation of mitochondria because it utilized 0.25 M sucrose and it was felt that the same concentration of sucrose should be used for the centrifugation of both liver and testes samples (142, 151). While using 0.88 m sucrose may produce mitochondria that are morpholog-

ically more intact than are those isolated at 0.25 M sucrose, other problems arise when using 0.88 M sucrose. These include (1) the structureless appearance of other SM components such as nuclei, (2) distortion and complete breakage of some mitochondria following the application of stains such as osmuim tetroxide and (3) considerable variation in the length and shape of the mitochondria (76). Finally, isotonic (0.25 M) sucrose is very commonly used in the isolation of mitochondria, likely because all subcellular components are kept relatively intact, the preparations are preserved better and appear more homogeneous, and there is no agglutination. However, some disruption of the outer membrane does occur (76, 142).

Phospholipid Determination

A good lipid extract must initially be achieved if phospholipids are to be separated properly by chromatographic means. This is relatively easy to accomplish with serum or plasma, but thorough homogenization or sonic disintegration is necessary for animal tissue or plants. Chloroform:methanol is commonly used to extract lipids, and the addition of a salt solution will remove non-lipid material such as amino acids, salt, sugars and water soluble phosphate esters (54). According to Folch et al (54) following the addition of a salt solution virtually no lipid remains in the upper portion. Care must be taken to avoid lipid peroxidation, or the phospholipids will not chromatograph as discrete spots. Marinetti (106) suggests that 10-30 μl is an ideal volume for spotting, since overloading often causes spot elongation. It is recommended that the plates be chromatographed soon after spotting, since lipids can undergo changes if left for even a few hours.

Humidity is an important factor in phospholipid chromatography. Letters (93) reports that humidity variations of as little as 1% can

cause either streaking or flattened spots.

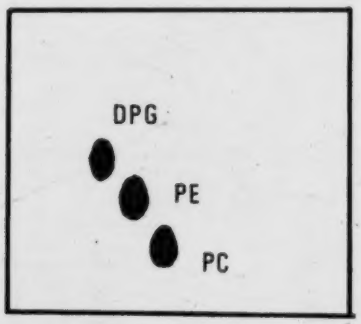
For phospholipid quantitation, most researchers (57, 63, 74, 81, 107, 125, 128, 129, 136, 139, 176) use a modification of Bartlett's method for phosphorus determination (6). In this method the scraped spot (absorbant plus sample) is first digested in perchloric acid and a reducing agent such as ascorbic acid or stannous chloride is added. The reagent used is ammonium molybate which specifically reacts with phosphorus. It is recommended that the amount of absorbant scraped for each sample to be digested be kept uniform (107).

Many satisfactory methods are available which describe the chromatographic separation of phospholipids. Most methods commonly used are one-dimensional i.e. the solvent front is run in one direction only. This is usually adequate for the separation of the more commonly measured phospholipids such as lecithin (PC) or phosphatidyl serine. In order to separate out acidic phospholipids such as cardiolipin (DPG) two-dimensional methods are required (57, 128, 176). This involves 1 or 2 additional runs where the solvent front is allowed to pass at right angles to the original solvent front. However, not all two-dimensional methods satisfactorily separate out acidic phospholipids. The method of Peter and Wolf (128), while two-dimensional, does not separate out cardiolipin. Getz et al (57) and Yavin and Zutra (176) describe a two-dimensional method which successfully separates out DPG. This is achieved by the addition of 40% methylamine. Acetic acid from the first phase has been found to interfere with the methylamine, however, washing with ether between the first and second directions alleviates this problem (57). In the method described by Yavin and Zutra (176), methylamine is used in the first direction, and it's presence in subsequent directions can lead to unsatisfactory separation of phospholipids. This problem is

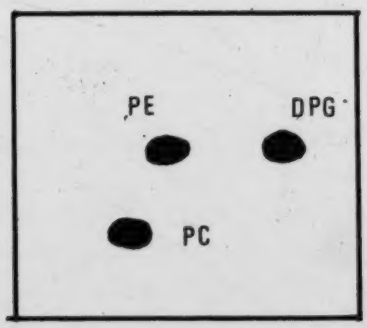
overcome by placing the plates in HCl fumes which neutralizes any traces of methylamine.

Most two-dimensional systems require the presence of an acid such as ammonia or acetic acid. Some researchers have suggested that these acids cause some hydrolysis of the phospholipids, but Marinetti (106) feels that this problem is negligible.

While the method of Poorthuis et al (129) was developed specifically for the separation of DPG and phosphatidyl glycerol, it was not chosen in this investigation because the 3 phospholipids of particular interest in this study (PC, PE and DPG) would have been extremely difficult to separate out (see figure below).



The method of Yavin and Zutra (176) was used in this investigation because, while being a sensitive micromethod, the plates used are of a sufficient size (10 x 10 cm) to allow for excellent separation of the 3 phospholipids concerned.



The method chosen for phosphorus determination was the micro-method of O'Brien et al (119) based on Bartlett's method. It was felt that the method selected should be specifically designed for detecting micro quantities of phosphorus, and the method of O'Brien et al (119) was known to be a very accurate and reproducible method.[1]

FOOTNOTES

1. Personal communication: George Chan, M.S., Department of Pediatrics, University of Alberta.

V. RESULTS AND DISCUSSION

A. Feeding Trial

Prior to initiation of the animal feeding trial all weanling rats appeared to be in good health. After 24 hrs of ad libitum feeding the mean intake of the control group was calculated to be 9.5 g, while the mean intake of the experimental group was 9.1 g. For the first 3 wks of the feeding trial the intake of each rat in the control group was limited to the intake[1] of it's pair in the experimental group. Pair-controlled feeding was discontinued after 3 wks when it was felt that a similar growth curve would be achieved on ad libitum feeding. Some control rats did not consume even the restricted amount of food.

A complete record of food consumption is presented in Appendix XIII, while a complete record of weight gain is presented in Appendix XIV. Figures 6 and 7 display the food consumption and growth curves respectively of the control versus experimental groups throughout the entire feeding trial period of 24 wks. The mean food intake of both groups was very close for the first 3 wks of the feeding trial. It is difficult to say whether this was due to the pair-controlled feeding or because the initial weights of the animals were so closely matched. The natural consumption of food does correlate well with metabolic size according to the lipostat theory (20). After the pair-controlled feeding was discontinued there was a period of 4 to 5 wks when the food consumption of the experimental group fell relative to the control group, but by week 8 this trend reversed. Perhaps the rats in the control group 'overate' initially to compensate for the restrictions imposed during the first 3 wks. Between weeks 3 and 9 there was continued growth in spite of a

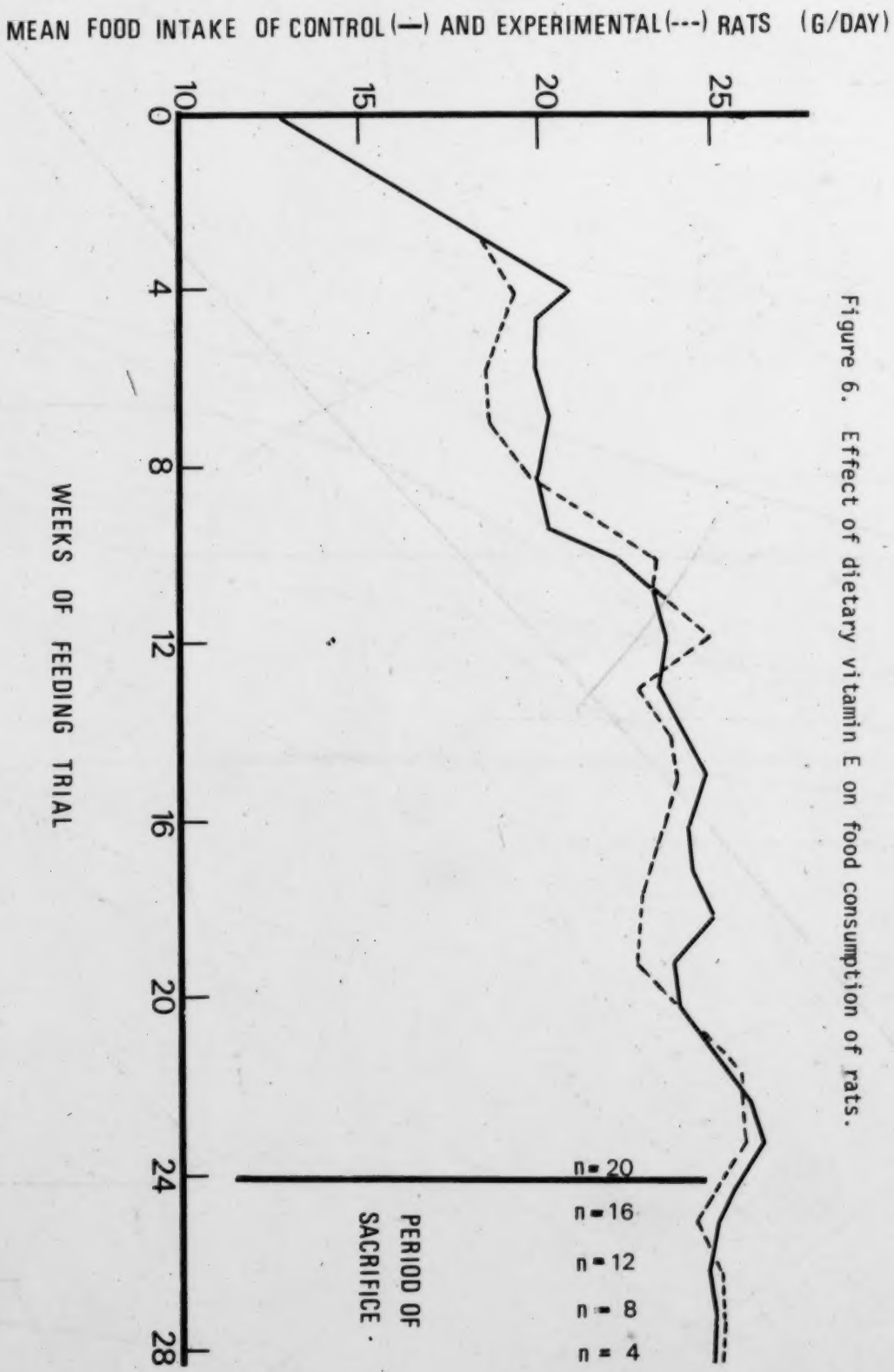
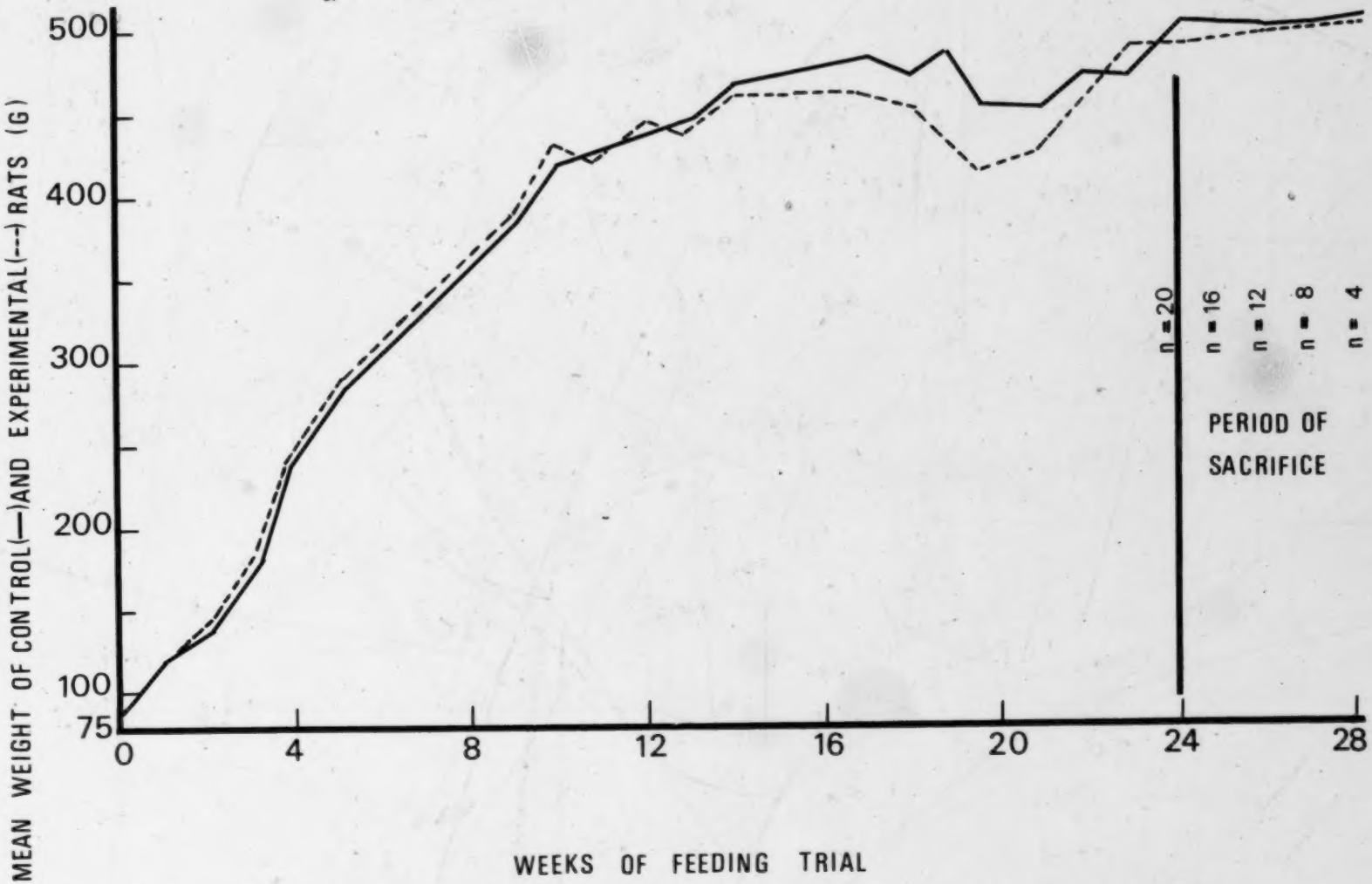


Figure 6. Effect of dietary vitamin E on food consumption of rats.

Figure 7. Effect of dietary vitamin E on growth of rats.



plateau in food consumption. Perhaps the rats consumed their feces (coprophagia) or were less physically active, thus conserving calories for growth.

The minor fluctuations in the food consumption curve, none of which were significant, were not reflected in the growth curve. The mean weights remained very similar throughout the first 18 wks of the feeding trial. Between weeks 18 and 19 the mean weight of the control group rose sharply while the mean weight of the experimental group dropped. At the beginning of week 19 the difference between the mean weights of both groups was statistically significant ($p < .01$) according to the unpaired t-test (Table 4). Unfortunately, within 2 to 3 days of week 19 the rats in the experimental group developed a diarrheal illness. Two to three days after the experimental animals developed diarrhea, the control animals developed what appeared to be the same illness. The cause of the diarrhea was not determined. Within a 1 wk period all animals from both groups were affected. The condition lasted for 3 to 4 days.

By week 20 all rats appeared to have recovered from the diarrhea illness. Between weeks 19 and 20 the mean weight of the control group dropped from 471 g to 444 g and remained at that level for a further week. For the vitamin E-deficient group, the decrease in mean weight which had become apparent prior to the diarrheal illness continued, and reached a minimum value of 406 g at week 20. From week 20 onwards there was a steady increase in weight gain. The difference in mean weight between the control and experimental group was statistically significant ($p < .01$) at week 20 as judged by the unpaired t-test (Table 4). The significance of the difference in mean weight between the control and

experimental groups disappeared after week 20. At the end of the feeding trial there was no significant difference in mean weight between the two groups.

Table 4. Effect of dietary vitamin E on growth of control and experimental rats.

Number	Initial Weight[a]		Weight at Week 19		Weight at Week 20		Final Weight[b]	
	C[c]	E[d]	C	E	C	E	C	E
1	63	65	415	404	398	388	469	472
2	67	67	464	419	429	406	484	501
3	71	70	477	471	462	451	508	509
4	72	70	424	373	395	340	464	426
5	73	73	484	412	450	380	530	473
6	75	73	424	436	376	404	470	486
7	78	75	485	432	449	400	516	483
8	78	77	489	413	453	381	534	454
9	79	79	483	443	446	413	521	505
10	80	83	436	394	414	357	483	472
11	81	84	457	511	433	468	506	562
12	83	84	450	428	442	386	508	483
13	83	85	471	427	435	402	503	504
14	84	86	466	432	441	386	518	504
15	85	88	481	447	462	422	491	487
16	88	88	522	385	498	366	569	474
17	90	96	488	484	469	457	531	549
18	91	97	478	492	461	470	512	541
19	96	98	516	451	479	424	518	503
20	98	105	502	445	487	421	538	496
MEAN	81	82	471	435*[e]	444	406*	509	494
SEM	9.2	11.1	29.4	35.3	30.9	35.7	26.3	31.6

a. Weight is expressed to the nearest g.

b. Represents weight at sacrifice, wks 24 - 28.

c. Indicates control group.

d. Indicates experimental group.

e. *Indicates significant difference between control and experimental group ($p < .01$).

The effect of vitamin E deficiency on growth has been investigated by others. The findings of Bieri et al (9), Carpenter (26), Grinna (60), Machlin et al (103) and Yang et al (175) are summarized and compared with my findings in Table 5. While 4 out of the 6 studies listed reported no effect of vitamin E deficiency on weight gain, those by Machlin et al (103) and Yang et al (175) found that weight gain was significantly lower for the vitamin E-deficient rats. In the latter studies, 10% stripped corn oil comprised the type and quantity of fat in the diet, as was the case with the present study. In half the studies where no significant difference in weight gain between the 2 groups is reported, the source of dietary fat is 3% or 4% stripped lard. Increasing the PUFA composition of the diet is known to increase the requirement of vitamin E (79, 173, 174) therefore a 10% stripped corn oil diet would be expected to have a more pronounced effect on any deficiency signs, such as low weight gain. Conversely, if the type of fat is saturated, and constitutes a small proportion of the total calories, the requirement for vitamin E would be lower and any deficiency signs caused by a low vitamin E intake might be suppressed.

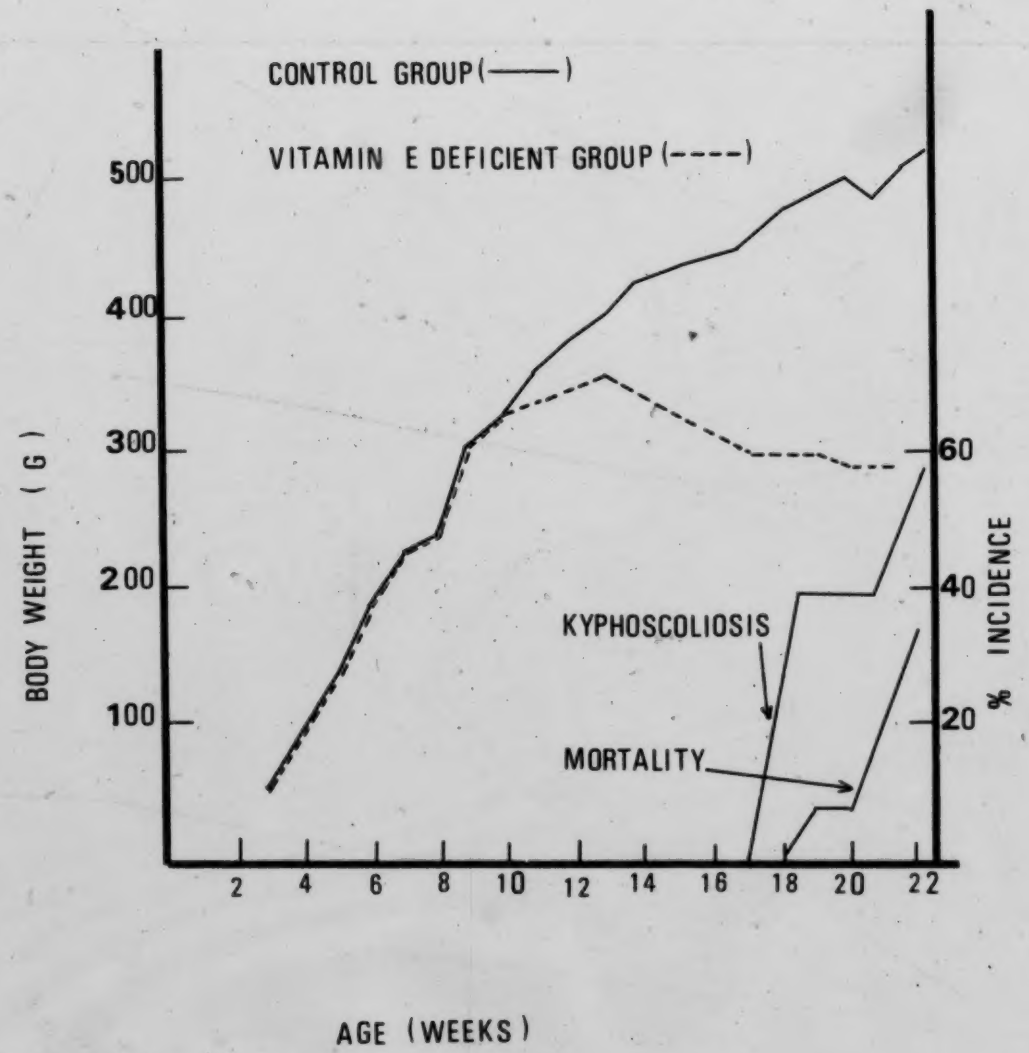
The growth curve and incidence of mortality of control and vitamin E-deficient rats as reported by Machlin et al (103) is illustrated in Figure 8. The growth curve of the control rats is very similar to that found in my study. In the latter 2 studies the weight of the control rats at age 22 wks was found to be approximately 500 g, and represents a much higher total weight gain than was found by other investigators (9, 26, 60) who maintained male rats for a comparable or greater period of time. In my study the growth curve of the experimental rats is very different from that reported by Machlin et al, in spite of a similar experimental design and growth curve for the control rats.

Table 5. Comparison of dietary vitamin E level, dietary fat composition and weight gain of vitamin E-deficient and vitamin E-supplemented rats.

Investigator	Study Period[a]	Tocopherol Supplementation[b]	Diet[c]	Final Weight[d] C[e]	Final Weight[d] E[f]	
This Study	4 to 24 - 28	250	10% stripped corn oil	509	494	NS[g]
Bieri et al (9)	3 to 16 - 20	200	4% stripped lard	"equally good" (exact figure not reported)		NS
Carpenter (26)	2 to 16		3% stripped	355	331	NS
	2 to 28	500	lard plus	380	375	NS
	2 to 56			417	450	NS
Grinna (60)	4 to 11	300	10% stripped	135	135	NS
	26 to 42		corn oil	410	422	NS
Machlin et al (103)	3 to 10	200	10% stripped	325	325	NS
	3 to 26		corn oil	475	273	*
Yang et al (175)	3 to 32	250	10% stripped corn oil	332	166	*

a. Expressed in weeks from start to finish.
b. Expressed in mg vitamin E per kg diet.
c. Describes type and quantity (% by weight) of fat.
d. Expressed in g to nearest whole g.
e. Indicates control group.
f. Indicates experimental group.
g. NS indicates not statistically significant
* Indicates statistically significant, $p < .01$.

Figure 8. Effect of dietary vitamin E on growth and mortality of rats(103).



According to Machlin et al, the difference in weight gain between the control and deficient male rats became statistically significant at 12 wks of age. In my study, a statistical difference in mean weight appeared at weeks 19 and 20. It might be argued that this difference was due to the diarrhea, since the vitamin E-deficient rats developed the diarrhea a few days before the control rats became sick. The degree of vitamin E deficiency present in the experimental rats at 18 wks may have compromised their immune system (146) making them more susceptible (ie. earlier onset and greater weight loss) to a possible viral illness. However, the decrease in weight became apparent before the experimental rats acquired the diarrhea. For a short period of time the mean weight of the experimental group was lower (apparently) in response to vitamin E deficiency and up to week 20 the growth curve resemble those in Figure 8.

An explanation for this major difference between the growth patterns of the experimental rats in the study by Machlin et al versus my study might be that the animals in my investigation had considerably greater vitamin E stores at the onset of the feeding trial. The weight and age of the animals at the beginning of any dietary regimen is an important factor in considering the effect of the diet on weight gain. The initial weight and age of the rats in the studies by both Machlin et al and Yang et al was 50 g and 3 wks of age. The animals at the onset of my experiment were 1 wk older, yet their mean weight was more than 50% greater. The same species of rats were used by Machlin et al and Yang et al. Growth of rats is very rapid in the first few weeks and in that period of time absorption and storage of tocopherol is significant (99). Perhaps the experimental animals in this study had sufficient tocopherol stores to produce similar results ie. no effect of

tocopherol-deficiency on weight gain, as was achieved by investigators who started a vitamin E-deficient diet early but used a more saturated diet (9, 26). This might explain why Grinna (60), who also fed a 10% stripped corn oil diet to male rats from 4 weeks of age, also did not find any effect of vitamin E deficiency on weight gain. Ames (2) has demonstrated that the requirement for vitamin E in female rats increases with age but this may not be the case for male rats. It is difficult to believe that a difference in vitamin E intake of only 1 wk in the infancy period could produce the difference in weight as just discussed. There must be other as yet identified factors in the genesis of vitamin E deficiency symptoms.

B. Physical Appearance

At the time of sacrifice, both control and vitamin E-deficient rats were examined for physical appearance. Several deficiency signs have been associated with tocopherol-deficiency including nasal porphyrin secretion[2] (103, 175), greasy-rough coat (175), kyphoscoliosis (103, 175), skin ulcerations (103), testicular degeneration (9, 26, 124) and paralysis (175). In this study only 2 such deficiency signs were noted, nasal porphyria and greasy-rough coat. Both signs were present in the control animals as well as the experimental animals. Table 6 lists the incidence of both nasal porphyria and greasy-rough coat in both the control and vitamin E-deficient rats. In order to objectively compare the incidence of the 2 physical signs, a statistical sign test was applied to the data. The sign test for statistical comparison of incidence of nasal porphyria between the control and vitamin E-deficient rats is described in detail in Appendix XV, while the sign test for statistical comparison of incidence of greasy-rough coat is described in detail in Appendix XVI. According to the sign test there was a significant difference in the

Table 6. Effect of vitamin E deficiency on presence[a] or absence[b] of nasal porphyria and greasy-rough coat.

Rat Number	Nasal Porphyria		Greasy-Rough Coat	
	C[c]	E[d]	C	E
1	-	+	-	-
2	-	-	-	+
3	-	+	-	-
4	-	+	-	+
5	-	+	-	+
6	-	-	-	-
7	+	-	-	-
8	+	+	+	-
9	-	-	-	+
10	-	+	-	-
11	-	+	-	+
12	+	+	-	-
13	-	+	-	-
14	-	+	-	+
15	-	-	-	-
16	-	-	-	-
17	-	+	+	-
18	+	+	-	+
19	-	-	-	+
20	+	+	-	-

a. Plus sign (+) indicates presence of clinical sign at time of sacrifice.

b. Minus sign (-) indicates absence of clinical sign at time of sacrifice.

c. Indicates control group.

d. Indicates experimental group.

incidence of nasal porphyria between the control and experimental groups, while there was no statistical difference in the incidence of greasy-rough coat between the two groups.

In this investigation, control rats also were affected with deficiency signs. The incidence of nasal porphyria was 65% in the vitamin E-deficient group and 25% in the control group. According to Machlin et al (103), porphyrin nasal secretion is difficult to differentiate from a hemorrhagic discharge of the nose, and may be present in the rat as a general reaction to stress. The incidence of porphyrin nasal secretion reported by Machlin et al was 25% for the vitamin E-deficient group and 0% for the control group. There was a fairly high incidence of kyphoscoliosis (32%) which is a rare finding in vitamin E deficiency experiments. In the latter investigation, kyphoscoliosis was noticed as early as 17 wks and correlated well with the incidence of mortality. Analysis of Figure 8 reveals that the skeletal abnormality started about 5 wks after there was a statistically significant difference in mean weight, or at approximately the same time as when a stabilization of weight loss occurred in the experimental group. Severe deficiency signs such as skeletal deformity and loss of weight were not seen in my study or those of others (9, 27, 60) presumably because very severe vitamin E deficiency did not occur at a critical time. Yang et al (175), who also demonstrated a significant decrease in weight in association with vitamin E deficiency reported the presence of deficiency signs similar to those described by Machlin et al, ie. leg paralysis, greasy-rough coat, bleeding nose and deformed skeleton.

In my study, 10% of the control rats and 40% of the experimental rats were found to have a greasy, rough coat. It is difficult to explain why 2 of the control rats developed this sign, which along with

nasal porphria, is one of the more commonly reported vitamin E deficiency signs. If one assumes that the presence of a greasy-rough coat in the 2 control rats is completely unrelated to vitamin E deficiency (their serum alpha-tocopherol levels were high[illegible]t could be explained by other factors such as too dry an atmosphere o[illegible]ermatitis-type response to the bedding. However, such factors could be applied to the experimental group. The exact incidence of greasy-rough coat is not reported by Machlin, perhaps because it is a difficult sign to measure objectively.

C. Blood and Tissue

The values for hematocrit levels are present in Table 7. There was no significant difference between the mean hematocrit reading of the 2 groups (46.0% for the control versus 45.6% for the experimental rats). The good hematocrit values were achieved in spite of the use (in the diet) of ferric and cupric salts (Appendix I), which are not absorbed as well as the reduced (ferrous cuprous salts) forms of iron and copper.

Tsen and Collier (163) also reported that dietary vitamin E had no effect on hematocrit levels. Their control rats had a mean hematocrit value of 49.6% compared with a mean of 49.5% for the vitamin E-deficient group. The diet used in their experiment was based on lard, but there was a significant difference in the degree of hemolysis i.e. 2.1% for the control group versus 98.5% for the experimental group. This is in contrast to the findings of Machlin et al (103) who found that a lowered hematocrit was observed in the experimental group (38.2% compared with 41.8% for the controls) from 16 wks of age i.e. after 13 wks on a vitamin E-deficient diet. As with the other deficiency signs reported by Machlin, the effect on hematocrit occurred after the decrease in weight.

Levander and associates (94-97), who have studied extensively the effect of vitamin E deficiency on blood parameters,[3] found no direct

Table 7. Effect of dietary vitamin E on hematocrit[a] levels of rats.

Number	Control Group[b]	Experimental Group[c]
1	49.5	43.5
2	47.5	49.0
3	48.0	47.0
4	45.0	40.0
5	47.0	43.0
6	46.5	-[d]
7	43.5	44.0
8	41.0	38.0
9	46.5	42.5
10	46.5	45.5
11	51.5	49.5
12	47.5	51.0
13	41.0	-
14	45.5	47.5
15	45.0	41.5
16	46.5	48.0
17	47.0	49.0
18	45.5	46.5
19	46.0	48.5
20	44.0	47.5
MEAN[e]	46.0 (NS)[g]	45.6
SEM[f]	2.48	3.60

a. Expressed as % packed RBC in volume of blood.

b. Supplemented with 250 mg dl-alpha-tocopherol acetate per kg vitamin E-deficient diet.

c. Vitamin E-deficient diet.

d. - indicates values not available.

e. Each value represents the average of two readings.

f. Standard error of the mean.

g. NS indicates no significant difference between the mean of both groups.

relationship between vitamin E deficiency and hematocrit value. However, the mean hematocrit value was lower in vitamin E-deficient lead-poisoned rats than in vitamin E-sufficient lead-poisoned rats.

Ausman and Hayes (4) report lower hematocrit levels (12 - 15%) in vitamin E-deficient cebus monkeys fed a 22%[4] safflower oil diet for 12 - 13 months. This was associated with plasma vitamin E levels of less than 0.1 mg/100 ml. Chou and associates (30, who maintained weanling male rabbits on a vitamin E-deficient diet for 4 wks, found that there was no significant difference in hematocrit levels between the control and vitamin E-deficient rabbits in spite of a lower level of plasma iron, a higher TIBC[5] and a higher reticulocyte count in the experimental group.

The results for tissue weight determination are listed in Table 8. There was no significant difference between the control and experimental groups in the mean weight of liver or testes tissue, as determined by the t-distribution. The values for tissue weight are compared with those determined by Carpenter (26), Papu et al (124) and Yang et al (175) in Table 9. The findings of my study agree with those of other investigators (55, 60, 144, 175) that vitamin E deficiency has no effect on liver weight. Yang and associates (1975) report that liver weight is increased in vitamin E deficiency only when reported on the basis of total body weight, but when liver weight is expressed as a raw score in g, there is no signficant difference between control and vitamin E-deficient rats.

Bieri and Andrews (9) fed 3 wk old weanling rats a 4% stripped lard diet with or without vitamin E for 20 wks. While growth was equally good between the 2 groups there was a significant decrease in the testicular weight of the deficient group. The magnitude of this dif-

Table 8. Effect of dietary vitamin E on rat liver and testes weight.

Number	Liver[a]		Testes[b]	
	C[c]	E[d]	C	E
1	8.93	9.37	4.15	3.37
2	9.96	8.79	3.77	3.70
3	10.56	11.02	3.96	3.99
4	10.17	9.84	4.01	3.67
5	11.01	9.74	4.06	3.78
6	9.95	9.53	3.33	3.27
7	9.47	9.86	3.92	4.30
8	9.97	9.81	3.93	4.41
9	9.83	9.73	3.61	3.29
10	10.49	10.19	3.50	3.49
11	10.23	9.62	3.95	3.98
12	9.85	10.36	3.76	3.70
13	10.43	10.41	3.93	3.98
14	10.27	10.17	3.56	4.00
15	9.98	10.13	3.92	4.37
16	10.20	9.96	4.07	3.73
17	9.73	9.99	4.02	3.68
18	9.99	10.48	3.48	4.00
19	9.79	10.38	3.46	3.47
20	10.07	10.21	3.84	3.29
MEAN	10.04	9.98 (NS)[f]	3.81	3.77 (NS)
SEM[e]	0.43	0.47	0.24	0.35

a. Values represent weight of total liver in g.

b. Values represent total weight in g of a pair of testes.

c. Indicates control group.

d. Indicates experimental group.

e. Standard error of the mean.

f. NS Indicates no significant difference between the mean of both groups.

Table 9. Comparison of liver and testis weights in vitamin E-deficient and vitamin E-supplemented rats.

Investigator	Study Period[a]	Diet[b]	Liver Weight[c]			Testis Weight[f]		
			C[d]	E[e]		C	E	
This study	4 to 24-28	10% Stripped Corn Oil	10.04	9.98	NS[g]	3.81	3.77	NS
Carpenter (26)	2 to 8	3% Stripped Lard				2.36	2.55	NS
	2 to 12					2.80	1.88	*[h]
Pappu et al (124)	2 to 24	8% Peanut Oil				1.50 (single testis)	0.90	*
Yang et al (175)	3 to 32	10% Stripped Corn Oil	8.20	8.80				NS

a. Expressed in weeks from start to finish.
b. Describes type and quantity (% by weight) of fat.
c. Expressed in grams.
d. Indicates control group.
e. Indicates experimental group.
f. Expressed as total weight in grams of a pair of testes.
g. NS indicates no statistical difference between the mean of each group.
h. * indicates a significant difference between the mean of each group ($p < .01$).

ference is not reported. Pappu and associates (124) maintained 2 wk old rats on an 8% peanut oil diet for 24 wks and demonstrated that there was a significant decrease in testes weight between the vitamin E-deficient and control groups. Peanut oil, while a vegetable oil, is much more saturated than corn oil. However, the dietary regimen was initiated when the rats were only 30 g in weight and the vitamin E stores of the rat would still be minimal. The weights reported by Pappu are weights for an individual testis. If these values are doubled to represent a pair of testes, the values (3.0 g for the control, 1.80 g for the experimental) are very close to those reported by Carpenter (26) ie. 2.80 g for the control group versus 1.88 g for the vitamin E-deficient group. Carpenter, in studying the effect of vitamin E deficiency on maturing rat testes found that there is an 8-fold increase in testes weight between 3 and 6 wks of age ie. from approximately 0.27 g to 1.95 g. This investigator also found that even though an inbred strain of rats was used in the experiment, there was a considerable variation present at 12 wks in the response of testes tissue to vitamin E deficiency. In some 3 mo old experimental animals the testes weight was comparable to the controls, while in others degeneration had occurred.

It is interesting to compare the body weights and testes weights versus age of the rats in my study with those values as reported by Carpenter. In the latter experiment, the rats were 16 days old at the beginning of the experiment, thus the initial vitamin E stores would be expected to be very low. The reported mean weight of the rats was 73 g, and at 6 wks, when most of the maturation process of the testes has occurred, the rats weighed 128 g. In my experiment, the mean age of the experimental rats at the onset of the feeding trial was 26 days (4 wks), but the mean body weight was 82 g. Sixty-five percent of the most rapid

testes growth had occurred on a vitamin E-rich rat milk diet, ie. pre-weaning. This likely corresponded to a significant level of vitamin E stores present in the experimental group at the beginning of the experiment. Also, the rats used in my experiment were known to be a particularly healthy colony of inbred rats produced by the Animal Sciences Department, University of Alberta. The robust constitution of the animals may have contributed to their resistance, while in other experiments the purchase of perhaps less healthy rats from a commercial breeding colony may have increased the susceptibility of the animals to vitamin E deficiency. There is no question that their weight for age was superior to that observed in other experiments. Grinna (60) who also used 4 wk old weanling male Sprague-Dawley rats, reported that at the beginning of the feeding trial[6] the mean weight was 63 g while at age 11 wks the mean weight of the control rats was 135 g, a weight which is very low when compared with the 11 wk weight of 423 g of the control rats in this experiment. A study of the mean weight versus age of control rats from the various investigators listed in Table 5 provides further support for this suggestion.

D. Tocopherol Status

Serum and tissue (liver and testes) alpha-tocopherol levels were determined in order to objectively assess the vitamin E status of the control versus deficient animals, to enable comparison of results with those of other investigators, and to draw more meaningful conclusions about the effect of vitamin E deficiency on the SM lipids.

The levels of serum and tissue alpha-tocopherol for the control and experimental rats are listed respectively in Tables 10 and 11. In the determination of alpha-tocopherol, the values for beta-carotene also are obtained, thus the values for beta-carotene are presented along with

Table 10. Effect of dietary vitamin E on rat serum alpha-tocopherol and beta-carotene levels.

Number	Serum Alpha-Tocopherol[a]		Serum Beta-Carotene[b]	
	C[c]	E[d]	C	E
1	1.41	0.16	116.7	126.7
2	1.57	0.04	114.8	111.1
3	1.90	0.10	113.7	118.2
4	1.53	0.19	114.6	112.5
5	1.50	0.19	120.8	112.5
6	1.53	0.18	117.9	116.7
7	1.48	0.17	126.7	113.3
8	1.30	0.19	115.6	114.4
9	1.53	0.19	118.0	115.6
10	1.10	0.13	108.9	113.3
11	1.36	0.10	112.5	106.3
12	1.49	0.09	127.8	106.7
13	1.60	0.19	115.6	114.3
14	1.28	0.18	123.1	122.2
15	1.31	0.30	114.4	115.5
16	1.11	0.08	102.7	115.5
17	1.24	0.21	112.0	117.6
18	1.40	0.24	106.7	111.1
19	1.10	0.12	106.8	115.3
20	1.43	0.13	111.1	116.7
MEAN	1.41	0.16[*f]	115.0	114.8
SEM[e]	0.19	0.06	6.4	4.6

a. Values expressed in mg alpha-tocopperol per 100 ml serum.

b. Values expressed in µg beta-carotene per 100 ml serum.

c. Indicates control group.

d. Indicates experimental group.

e. Indicates standard error of the mean.

f. * Indicates a significant difference between the mean of both groups ($p < .001$).

Table 11. Effect of dietary vitamin E on rat liver and testes tissue alpha-tocopherol and beta-carotene levels.

	Alpha-Tocopherol[a]				Beta-Carotene[b]			
	Liver Tissue		Testes Tissue		Liver Tissue		Testes Tissue	
Number	C[c]	E[d]	C	E	C	E	C	E
8, 13	37.0	9.5	17.5	9.2	130	128	130	163
7, 15	24.7	12.7	50.5	4.7	133	130	161	137
4, 5	57.8	10.1	17.5	6.6	122	122	118	140
14, 18	42.0	12.4	18.0	5.8	113	132	122	119
1, 20	32.9	9.3	27.5	5.4	127	128	132	124
16, 17	24.7	10.9	12.0	9.3	130	128	105	126
3, 11	22.3	9.1	21.2	9.4	133	129	130	180
2, 12	27.5	9.8	26.8	3.7	104	127	148	136
10, 19	24.3	7.7	12.2	8.1	114	122	112	120
6, 9	20.7	9.1	18.0	6.8	121	120	118	111
MEAN	31.4	10.1*[f]	22.1	6.9*	122.7	126.6	127.6	135.6
SEM[e]	11.5	1.5	11.2	2.0	9.8	3.9	16.8	21.3

a. Values expressed in µg alpha-tocopherol per g whole tissue.
b. Values expressed in µg beta-carotene per g whole tissue.
c. Indicates control group.
d. Indicates experimental group.
e. Indicates standard error of the mean.
f. * Indicates a significant difference between the mean of both groups ($p < .001$).

the values for alpha-tocopherol.

The mean value for serum alpha-tocopherol was found to be 1.41 mg per 100 ml for the control group and 0.16 mg per 100 ml for the vitamin E-deficient group. According to the unpaired t-test the difference between the 2 groups was statistically significant ($p < .001$). There was no significant difference between the 2 groups in the levels of beta-carotene. Similar results were obtained for the liver and testes tissue, but the differences between the control and experimental groups were less pronounced than were the values for serum, even though the differences were equally significant from a statistical standpoint ($p < .001$).

These results indicate that the experimental animals were vitamin E-deficient relative to the control animals, but the question must be raised as to whether the deficiency state produced was severe in comparison with that produced by other investigators. The data for serum alpha-tocopherol is compared with that of Barker et al (5), Bieri et al (14, 15, 19) and Machlin et al (103) in Table 12, and in Table 13 the results for tissue alpha-tocopherol are compared with the values obtained by Grinna (60) and Pappu et al (124). The mean serum alpha-tocopherol concentration in the control group is close to the level reported by Machlin et al, the slightly higher value might be explained by the additional vitamin E supplementation ie. 50 mg per kg diet added to the diet mixture, and the increased stores at the onset of the feeding trial. The higher value of 1.87 mg per 100 ml reported by Bieri et al (14) may have reflected a decreased physiological need for vitamin E because of the more saturated dietary fat.

The serum alpha-tocopherol level of the vitamin E-deficient group was approximately 2.5 times greater than the level obtained by Machlin et al (103) and 4 times greater than the deficiency state obtained by

Table 12. Comparison of serum alpha-tocopherol levels in vitamin E-deficient and vitamin E-supplemented rats.

Investigator	Study Period[a]	Diet[b]	Supplementation of Controls[c]	Serum Alpha-Tocopherol[d] C[e]	E[f]	
This Study	4 to 24	10% Stripped Corn Oil	250	1.41	0.16	p<.001
Barker et al (5)	not mentioned	10% Stripped Corn Oil	-	-	0.04	-
Bieri et al (14)	7 to 15	5% Stripped Lard	200	1.87	-	
Bieri et al (15)	3 to 9	5% Stripped Lard	-	-	0.08	-
Bieri et al (19)	3 to 15	10% Corn Oil	-	-	0.10	
Machlin et al (103)	3 to 29	10% Corn Oil	200	1.28	0.06	p<.001

a. Expressed in weeks from start to finish.
b. Describes type and quantity (% by weight) of fat.
c. Expressed in mg alpha-tocopherol per kilogram of diet.
d. Expressed as mg per 100 ml.
e. C indicates control group.
f. E indicates experimental group.

Table 13. Comparison of tissue alpha-tocopherol levels in vitamin E-deficient and vitamin E-supplemented rats.

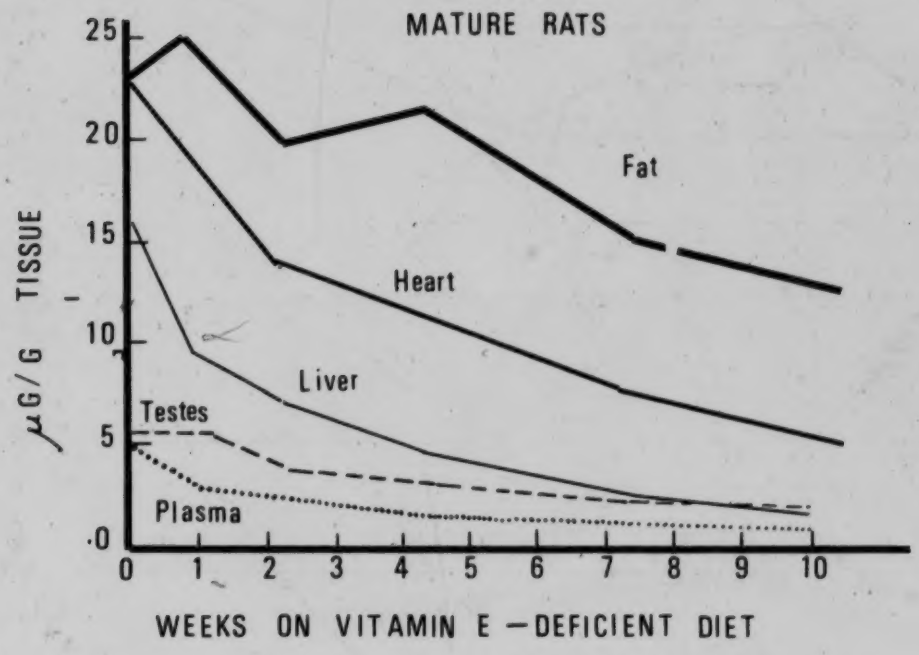
Investigator	Study Period[a]	Diet[b]	Supplementation of Controls[c]	Tissue Alpha-Tocopherol[d] Liver C[e]	Liver E[f]	Testis C	Testis E
This Study	4 to 24	10% Stripped Corn Oil	250	31.4	10.1*	22.1	6.9*
Grinna (60)	4 to 11	10% Stripped Corn Oil	300	23.0	2.40 *		
Pappu et al (124)	3 to 20	8% Stripped Peanut Oil	150	200.0	1.50*[g]		

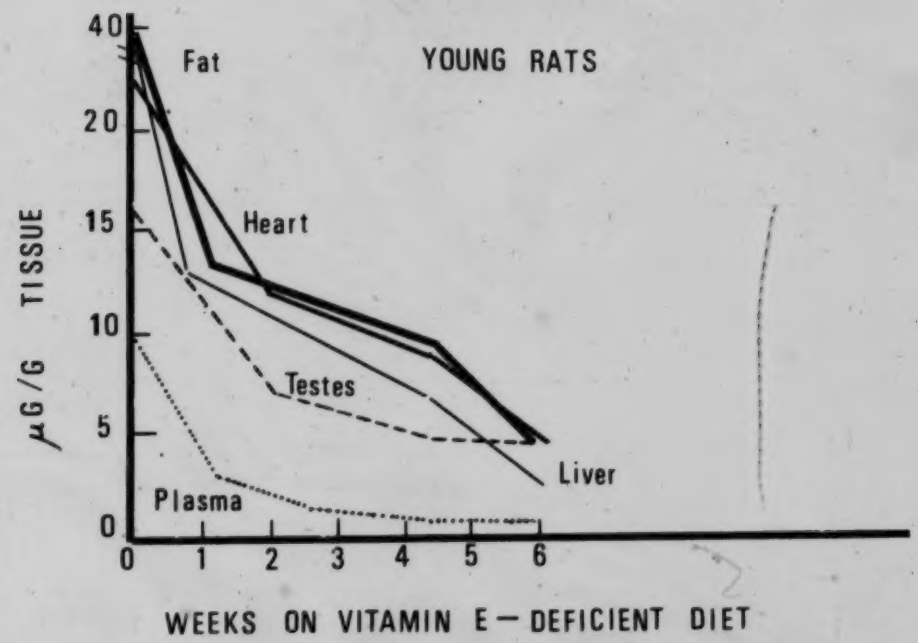
a. Expressed in weeks from start to finish.
b. Describes type and quantity (% by weight) of fat.
c. Expressed in mg alpha-tocopherol per kilogram of diet.
d. Expressed in µg alpha-tocopherol per gram of whole tissue.
e. C indicates control group.
f. E indicates experimental group.
g. * indicates a significant difference between the mean of both groups ($p < .001$).

Barker et al (5), both of which were also on a 10% corn oil diet. Wnen compared with the mean serum alpha-tocopherol level of animals who had been maintained on a vitamin E-deficient 5% stripped lard diet for a shorter period of time (15) the value is still higher, i.e. 2 times greater. One explanation for this could be the factor which has already been discussed, the possibility of greater vitamin E stores at the onset of the feeding trial.

The diarrheal illness at 19 wks, with its associated temporary bowel damage, might be expected to result in lowered vitamin E levels due to malabsorption. However, since the diet in question contained no vitamin E, malabsorption could not be the cause of the changed serum levels of vitamin E. One effect of diarrhea might be to mobilize a pool of "bound" vitamin E by mobilizing stored fat as a source of calories during a period of fat malabsorption. Bieri (17), in studying the depletion of vitamin E from various tissues, found that fat, which contains the greatest concentration of vitamin E, is depleted at a moderate to slow rate throughout the depletion period, compared with liver tissue, which loses about one-half the total vitamin E initially, then undergoes a slower rate of depletion; and testes tissue, which retains its vitamin E for 1 week, then loses about one-third in the second week, and finally undergoes a very gradual decrease. Figure 9 (17) compares the depletion of vitamin E from the liver, testes, plasma and fat tissue of five week old rats[7] versus mature rats[8] fed a vitamin E-deficient diet. The dramatic decrease in vitamin E levels which occurs in the fat and liver tissue of the young rats may partly be explained by growth (increased tissue mass), diluting the vitamin E. However, the prolonged withdrawal of vitamin E from fat is apparant also in the mature rats. While adipose (fat) tissue vitamin E may not

Figure 9. Depletion of vitamin E concentration from tissues of mature versus young rats fed a vitamin E-deficient diet(17).





be sufficiently available to maintain plasma vitamin E at normal levels, or to prevent severe signs of vitamin E deficiency (17, 104), it may be released in large enough quantity to maintain a low level of plasma vitamin E.

A third explanation for a higher than expected serum alpha-tocopherol level observed in the experimental group might be that the laboratory values were falsely high readings. Other investigators (5, 14, 15, 19, 103) did not report the values for serum beta-carotene, which are an intrinsic component to the measurement of alpha-tocopherol. When determining alpha-tocopherol, the concentration of alpha-tocopherol cannot be interpolated from the vitamin E standard curve because at 510 mμ there is also interference from beta-carotene. Three standard curves are required for the spectrophotometric determination of alpha-tocopherol. One standard curve of beta-carotene at 455 mμ measures the true value of beta-carotene. The second standard curve of beta-carotene, at 510 mμ, measures how much beta-carotene can be detected at that higher wavelength. The third standard curve comprises the alpha-tocopherol curve at 510 mμ. When an unknown sample is read in the spectrophotometer, the reading at 510 mμ represents the concentration of alpha-tocopherol plus an unknown amount of beta-carotene. This latter value can be calculated by comparing the beta-carotene values at 455 mμ and 510 mμ (see Appendix VII).

In the present experiment, the levels of serum beta-carotene are 115.0 μg per 100 ml and 114.8 μg per 100 ml respectively for the control and experimental groups. Because there was no significant difference in beta-carotene between the 2 groups, a greater proportion of beta-carotene was present in the experimental group relative to the concentration of alpha-tocopherol. Perhaps this increased interference resulted in higher than true values for alpha-tocopherol. Farrell (50) found that

both vitamin A and carotene levels were increased with high serum alpha-tocopherol concentrations. While the increase in vitamin A could be correlated directly with the increase in serum alpha-tocopherol, the increase in carotenoids could not. If there is no direct correlation between serum alpha-tocopherol and carotene concentrations, variations in carotenoid levels could affect the outcome of serum alpha-tocopherol determinations. This would be particularly true for liver tissue where carotene is stored.

Finally, there is a possibility that the experimental diet mixture contained vitamin E (contamination), providing a fourth explanation for the higher than expected values for serum vitamin E. To eliminate this doubt, an analysis of the powdered food should have been conducted before beginning the feeding trial. Vitamin E is excreted in the feces but it is not manufactured in the intestine (99, 112), therefore the practice of coprophagia would have provided a source of vitamin E only if the food previously had contained vitamin E.

The unusual nature of vitamin E deficiency is exemplified by the fact that vitamin E-deficient rats can have a serum alpha-tocopherol concentration as low as 0.1 mg per 100 ml without exhibiting any deficiency signs (19), while higher levels such as those found in my study and by others (49, 60) were associated with deficiency signs. Grinna (60) reported no effect of tocopherol deficiency on growth, testes weight or physical appearance, in spite of low liver alpha-tocopherol levels and a high (83%) rate of RBC hemolysis. In my study, the concentration of liver alpha-tocopherol in the control group is only 3 times higher than the level present in the experimental group, in comparison with a ratio of 10 to 1 in the study by Grinna, or a ratio of 9 to 1 for the serum levels of this study. Similar explainations could be used for this

observation as for the serum alpha-tocopherol levels, i.e., higher vitamin E stores, an effect of the diarrhea on the mobilization of vitamin E from tissues, interference by beta-carotene in the laboratory analysis, or contamination of the deficient diet mixture.

According to Bieri et al (15) a rat serum vitamin E level of 0.08 mg per 100 ml is associated with 93% hemolysis, while 84% in vitro memolysis occurs with a serum vitamin E level of 0.23 mg per 100 ml. Hemolysis is not significantly reduced (0 to 8%) until a serum vitamin E level of 0.39 mg per 100 ml is reached. According to these criteria, one would expect the experimental rats in my study to have a significant degree of hemolysis. While hemolysis was not measured, the high hematocrit values would seem to indicate that either hemolysis was not present, or that production of RBC's was increased (high recticulocyte count). Tsen and Collier (163) found a significantly higher rate of hemolysis in vitamin E-deficient rats in spite of normal hematocrit values. As already pointed out, hemolysis is not necessarily a good indicator of vitamin E status (33, 117).

The relatively high concentration of vitamin E in the testes of the experimental animals would explain why there was no decrease in testes weight. The relatively high values might suggest that the testes had preferential retention of vitamin E and were spared in some way. Presumably, during puberty of the rat, during the rapid growth phase of the testes, there was adequate vitamin E from stores. Perhaps a 'critical period' in the testes development had already occurred, causing a more generalized effect, indicated by signs such as nasal porphyria. Youneszai et al (178), when studying glucose metabolism in vitamin E-deficient rats there was a significantly lower ability to synthesize citrate.[9] They noted that this occurred after one month of consuming the experimental

diet at a time when no degenerative changes (gross or histological) were apparent in the testes. Thus, there is an indication that the metabolic effects of vitamin E deficiency can be detected prior to any changes in tissue weight.

E. Subcellular Membrane Analysis

Subcellular membrane (SM) protein was evaluated in order to assess any possible differences between the SM protein of the control and experimental groups, to express the phospholipid data as a ratio of lipid to protein, and to help determine, along with electron microscopy, the type(s) of SM isolated.

The values for liver and testes SM pellet weight per g whole tissue are presented in Table 14. There was no significant difference in pellet weight between the control and experimental groups as evaluated by the unpaired t-test. This indicates that there was no difference in the amount of SM present (ie. the fraction as isolated by the particular method used in this experiment) between the 2 groups. In Table 15 the values for SM protein are presented for the control and experimental groups. The level of testes SM protein for the control group appeared to be slightly higher than for the vitamin E-deficient group but this difference was not statistically significant as judged by the unpaired t-test. These results are in agreement with the findings of Grinna (60), but are opposite to those of Schwarz (144), who found that in vitamin E deficiency the content of mitochondrial protein per g liver tissue is reduced.

The values for SM protein agree very closely with the experimental values obtained by Ricquier et al (136) who studied rat brown adipose tissue mitochondria[10]. These investigators obtained a value of 8.5 mg mitochondrial protein per g whole tissue as determined by the

Table 14. Effect of dietary vitamin E on rat liver and testes subcellular membrane pellet weight.

Number	Liver Tissue[a]			Testis Tissue[a]	
	C[b]	E[c]		C	E
8, 13	0.17	0.23		0.16	0.13
7, 15	0.31	0.16		0.17	0.16
4, 5	0.15	0.23		0.14	0.12
14, 18	0.14	0.17		0.16	0.17
1, 20	0.13	0.17		0.12	0.12
16, 17	0.20	0.13		0.11	0.16
3, 11	0.14	0.22		0.14	0.10
2, 12	0.26	0.21		0.13	0.14
10, 19	0.17	0.27		0.10	0.11
6, 9	0.16	0.23		0.12	0.12
MEAN	0.183	0.202	NS[e]	0.135	0.133 NS
SEM[d]	0.059	0.043		0.023	0.024

a. Values are expressed as g subcellular membrane pellet weight per g tissue.

b. Indicates control group.

c. Indicates experimental group.

d. Indicates standard error of the mean.

e. NS Indicates no statistical difference between the mean of both groups.

Table 15. Effect of dietary vitamin E on rat liver and testes subcellular membrane protein levels.

Number	Liver[a]		Testis[a]	
	C[b]	E[c]	C	E
8, 13	5.2	5.4	4.1	3.9
7, 15	7.8	8.0	5.3	5.3
4, 5	5.3	9.4	8.2	9.6
14, 18	10.1	5.1	8.0	5.6
1. 20	5.8	5.3	10.6	6.0
16, 17	5.4	6.0	8.3	8.7
3, 11	9.7	10.0	8.3	5.6
2, 12	5.2	5.3	5.7	2.8
10, 19	5.1	5.4	6.1	6.3
6, 9	5.6	5.1	6.2	5.9
MEAN	6.52	6.50 NS[e]	7.08	5.97 NS
SEM[d]	1.95	1.90	1.92	1.99

a. Expressed as mg subcellular membrane protein per g whole tissue.
b. Indicates control group.
c. Indicates experimental group.
d. Indicates standard error of the mean.
e. NS Indicates no statistical difference between the mean of both groups.

biuret method. While studying rat liver mitochonria, Caplan and Greenawalt (24) use either the biuret or the Lowry method for determining protein levels, and report that either method produces similar values, thus the values obtained by Ricquier et al could be compared directly to those obtained in this investigation. Ricquier et al point out that their experimentally determined value comprises about 20% of the true mitochondrial protein content as corrected theoretically by enzyme assay of the mitochondrial fraction. Such results are substantiated by similar values for mitochondrial protein obtained by Reith et al (134) and Schwarz (144) who studied rat liver. The values obtained by Colbeau et al (34) were much higher (12 mg mitochondrial protein per g liver), while Guerra (63) has obtained experimental values as high as 15 mg mitochondrial protein per g rat liver.

The negative staining procedures outlined in Appendix VIII permit morphological identification of the isolated SM. The quality of isolated mitochondria can be assessed through the phospholipid to protein ratio, their ability to swell in the presence of phosphate, their respiratory activity and their ultrastructure (63). Similarily, contamination can be assessed by assay of enzymatic activities (eg. glucose-6-phosphatase to detect microsomes) and by morphological analysis (24, 63, 76, 160). One common contaminant when isolating SM is glycogen, however in the present study measures were undertaken (overnight fast) to deplete the liver of its glycogen supply (24, 63, 76, 139, 142). In the initial centrifugation step, the nuclei and RBC's usually are removed because of their heavier weight and different sedimentation rate (142). The most common mitochondrial contaminants are endoplasmic reticulum (ER) and microsomes (63, 126). This is particularly true when membrane disruption has occurred (126). The presence of mitoplasts

(inner mitochondrial membrane plus matrix) are "valuable indicators in evaluating the success in the fractionation procedure"[11] because they indicate that separation of the inner versus outer mitochondrial (OM) membrane has occurred to a significant degree. Mitoplasts are characterized by pseudopod-like extensions of the inner membrane (IM) and matrix (see Plate 2) and "the ability to undergo respiration-dependent configurational changes from orthodox (expanded) to condensed (contracted)"[12]. The outer mitochondrial membrane is characterized by a low protein concentration (0.75 to 1.0 mg mitochondrial protein per g whole tissue) and a smooth morphology (63). According to Hogeboom et al (76) the final mitochondrial pellet was distinctly yellow in color, while Parsons (126) in concentrating OM from swollen mitochondrial preparations describes the OM pellet as a pale yellow-brown color. The latter investigator, in attempting to increase the yield of OM by light sonication, found that breakage of the IM occurred, resulting in contamination of the OM with fragments of IM. The negative staining procedure itself is associated with significant membrane disruption, permiting only crude identification of membrane structures. The presence of white, ribbon-like bands is associated with ER while white, tubular structures identify broken IM structure. It is often difficult to differentiate between the latter two forms[13] (126, 143).

In my experiment, the pellets isolated were a yellow-beige color. Negatively-stained preparations of liver and testes SM from a control rat are illustrated in Plate 3. In the upper photograph (liver) the presence of mitoplasts is apparent, while in the lower photograph (testes) a mitoplast has been negatively-stained in the 'condensed' configuration, and the small pseudopod-like extensions are apparent. This provides strong evidence that a substantial proportion of the SM isolated

Plate 2 . (2) Electron micrograph of freshly prepared mitoplasts illustrating characteristic pseudopod-like extensions of the inner membranes and matrix. Fixed with 3% glutaraldehyde followed by 1% OsO_4 stained with uranyl acetate and lead citrate. x 25,000. (3) As above during orethodox (expanded) configuration. Note condensed (contracted) configuration in one mitoplast. x 60,000 (reprinted from reference 63).

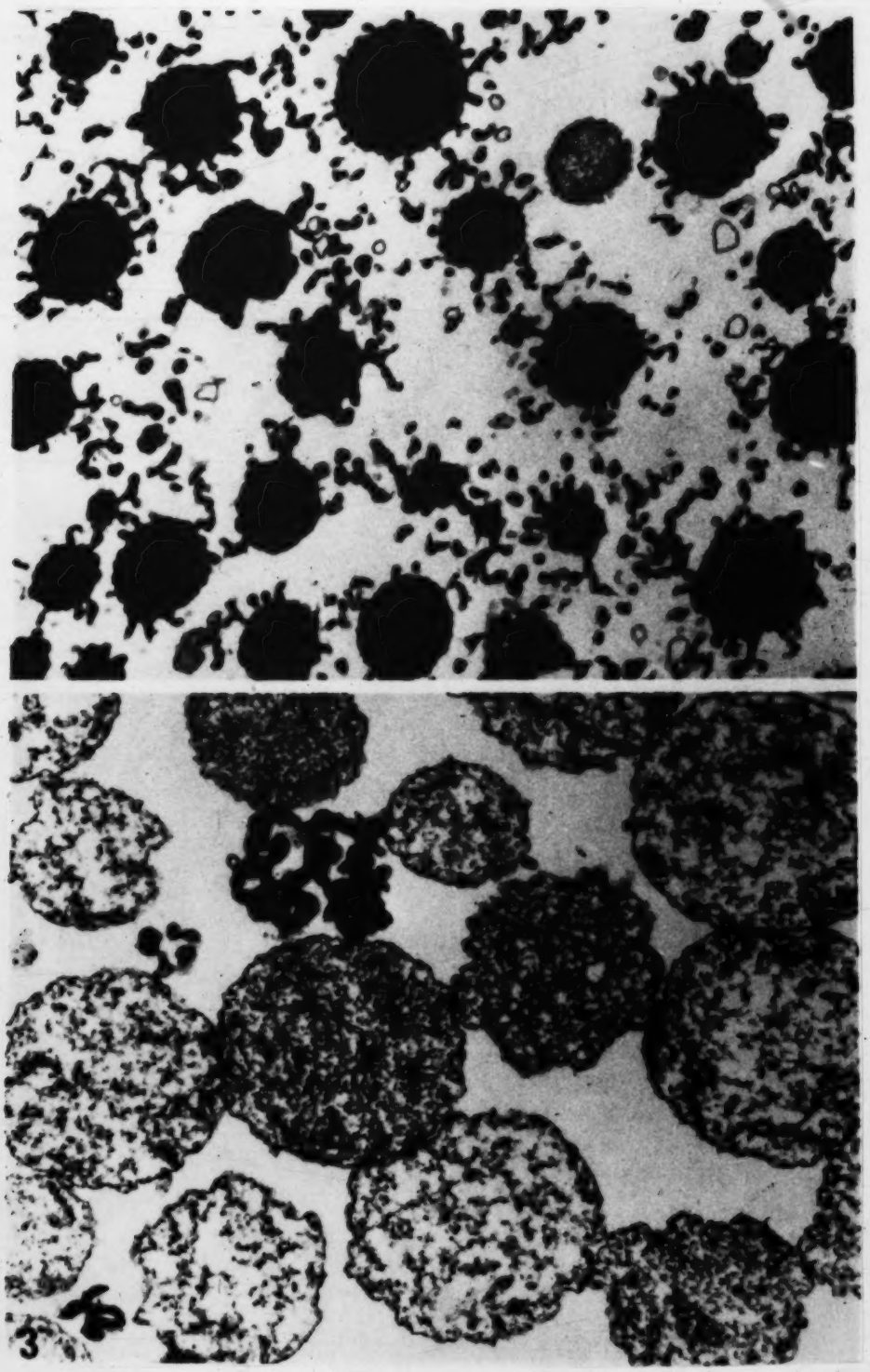
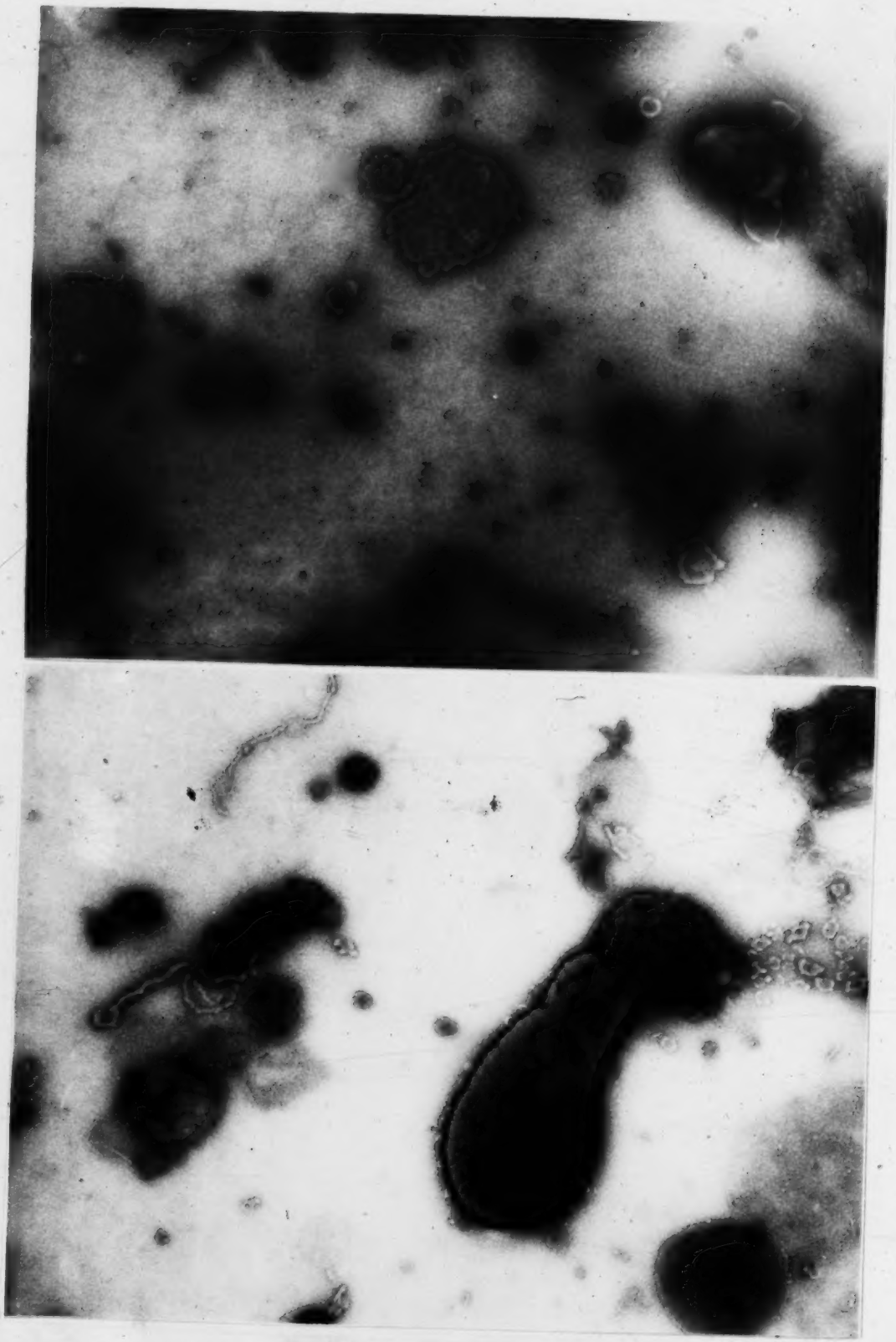


Plate 3 (a) Negatively-stained preparation of liver subcellular membrane from a control rat. Note presence of mitoplasts with pseudopod-like projections. x 40,000. (b) Negatively-stained preparation of testis subcellular membrane from a control rat. Note 'condensed' configuration. x 75,000.



was mitochondria. However, microsomal and ER membrane was evident in many of the negatively-stained preparations. Plate 4 depicts microsomal and ER contamination of mitochondria from a negatively-stained sample of testes SM.

In this investigation there were no differences observed with respect to the surface density of SM. Plates 5 and 6 respectively illustrate a negative stain of testes SM from a control versus a vitamin E-deficient rat. While the surface density appears similar, the tubular membrane network (cristae) of the IM is more evident in the control rat.

These findings are in contrast to the findings of Molenaar et al (115). However, Molenaar observed that the OM of the mitochondria was the most adversely affected membrane, while the SM illustrated in Plates 5 and 6 most likely represent mitoplasts (IM plus matrix).

Frigg et al (55), while studying the ultrastructural and stereological affect of vitamin E on mice liver mitochondrial membranes, observed that in the vitamin E-deficient mice there was a significant increase in surface density of the IM membrane. The surface to volume ratio of the IM of the mitochondria was found to be higher in the vitamin E-deficient group than in the control group. These investigators correlated their data with that of Schwarz (144) to show that, while the formation of the mitochondrial IM is enhanced in vitamin E deficiency, the cytochrome density is reduced. On the basis of negative stains of the SM isolated in this experiment, it cannot be concluded that there is any difference in the density or volume of the IM.

Heffron et al (73) studied the ultrastructure of skeletal muscle mitochondria from vitamin E-deficient (dystrophic) and control rabbits. The characteristics of the deficient mitochondria included swelling, loss of matrix and fragmentation of cristae (see Plate 7).

Plate 4 Negatively stained preparation of testes subcellular membrane from a control animal. Mitochondria (the matrix of the inner membrane has stained deeply and several broken outer membranes are visible, still attached to the inner membrane) account for approximately 80% of the subcellular membrane present in the photo. The ribbon-like bands likely are ER, while the shorter, tubular bands usually indicate free IM. The small pieces of membrane are 'unidentifiable artifacts' and likely represent microsomal membrane. x 15,000

Plate 5 Negatively-stained preparation of testes subcellular membrane from a control rat. x 200,000

Plate 6 Negatively-stained preparation of testes subcellular membrane from a vitamin E-deficient rat. No decrease in membrane surface density was observed. x 200,000

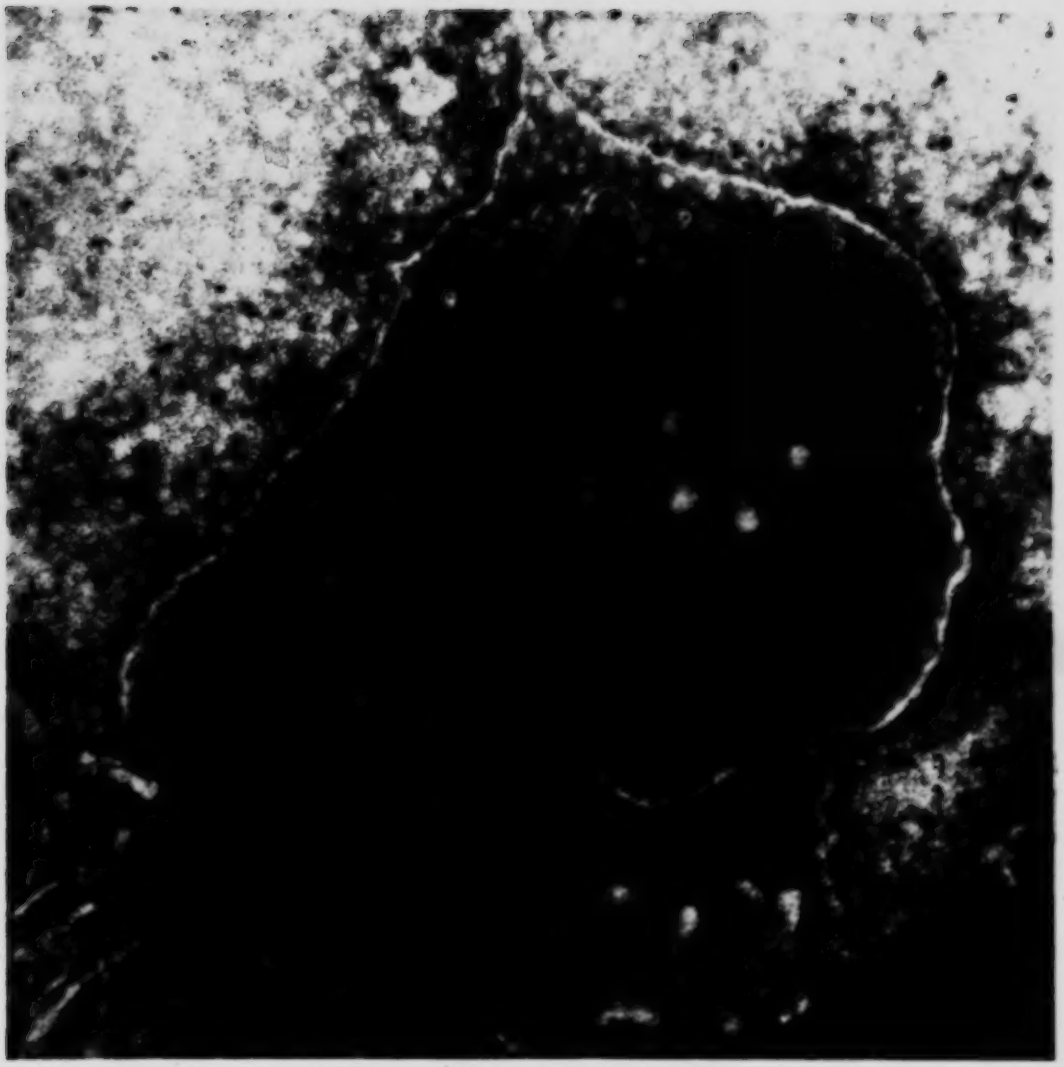
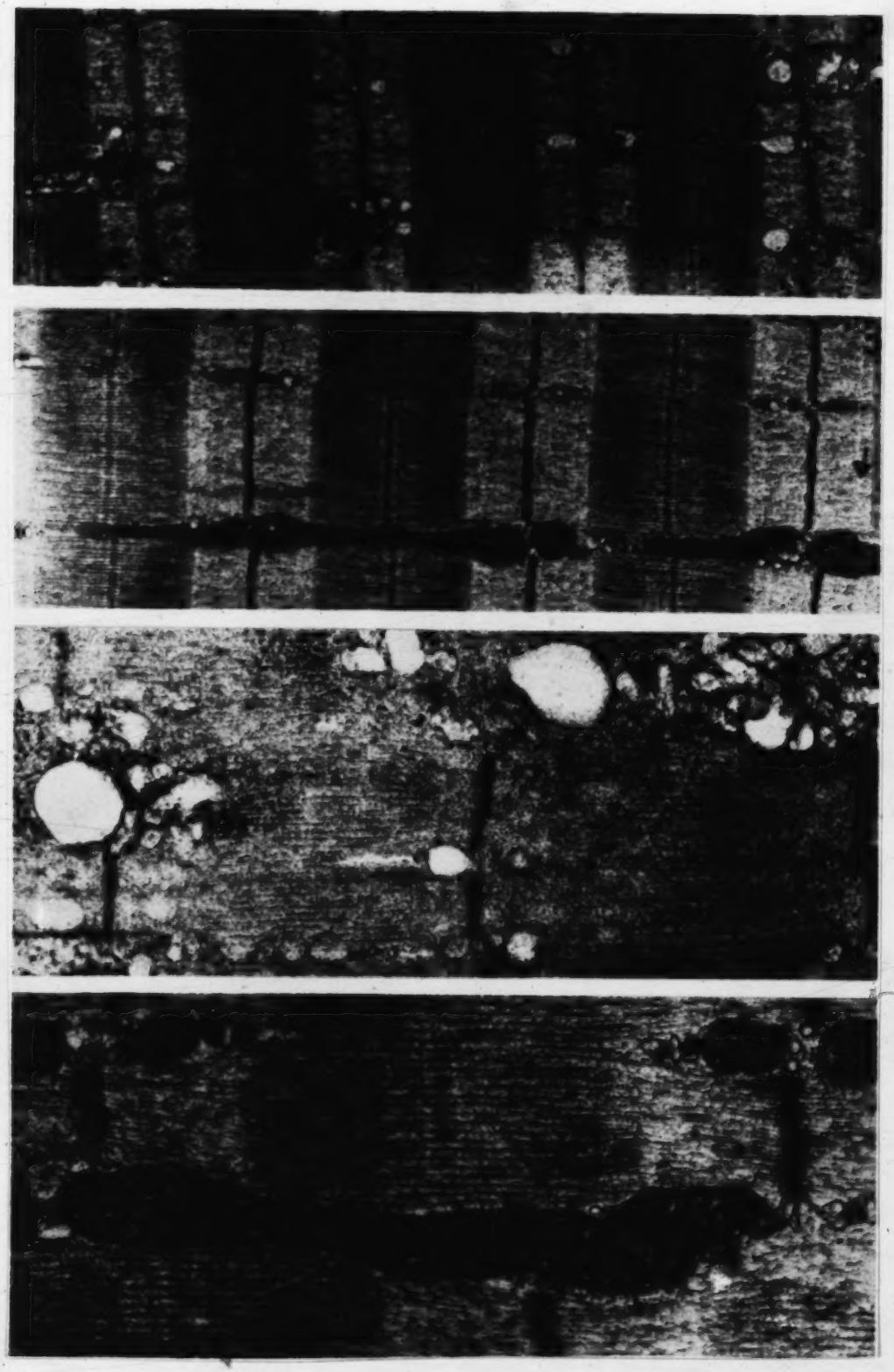


Plate 7 (a) Gastroenemius muscle from control rabbit fed on vitamin E-supplemented diet showing intact fine structure of mitochondria. x 42,600 (b) Dystrophic gastroenemius muscle from rabbit fed a vitamin E-deficient diet. Note the loss of matrix and fragmentation of cristae. x 22,500 (c) Rehabilitated gastroenemius muscle after vitamin E supplementation, showing mitochondria with intact fine structure. x 15,000 (d) Rehabilitated gastroenemius muscle after vitamin E supplementation. Localized areas still reveal abnormal mitrochondria similar to (b). x 12,000 (reprinted from reference 73).



The respiratory rate[14] of the dystrophic mitochondria was found to be severely reduced, but in animals that were rehabilitated with vitamin E, the respiratory rates were found to be normal. Similarily, the ultrastructure of the mitochondria from the rehabilitated rabbits displayed a normal appearance except for certain isolated areas which showed incomplete recovery. The evidence presented by Heffron that the biochemical changes caused by vitamin E deficiency could be restored following repletion with the vitamin was supported by Carabello (25).

In my investigation, similar differences as those observed by Heffron (ie. swelling, loss of matrix and fragmentation of cristae) were observed between the control versus vitamin E-deficient liver SM. These differences are illustrated in Plates 8 and 9. It must be pointed out that the electron microscope samples prepared by Heffron were 'fixed'[15] while those illustrated in Plates 8 and 9 represent negative stains. The negative stain of the vitamin E-deficient SM may be illustrating an artifact, such as the response of the SM to some component of the negative-staining procedure rather than a true representation of the membrane itself. An example of this is large amplitude swelling of mitochondria such as occurs in high sucrose concentrations, and is well described by Parsons (126) in Figure 10. There was a consistent difference between the photomicrographs of normal SM (mitochondria) (Plate 8) and vitamin E-deficient SM (mitochondria) (Plate 9). While this difference might partly be explained by artifacts related to changes produced during extraction or staining, the fact that both tissues underwent identical processing presumably means that the differences are due to the vitamin E content of the tissues.

Plate 8 Negatively-stained preparation of liver subcellular membrane from a control rat. x 50,000

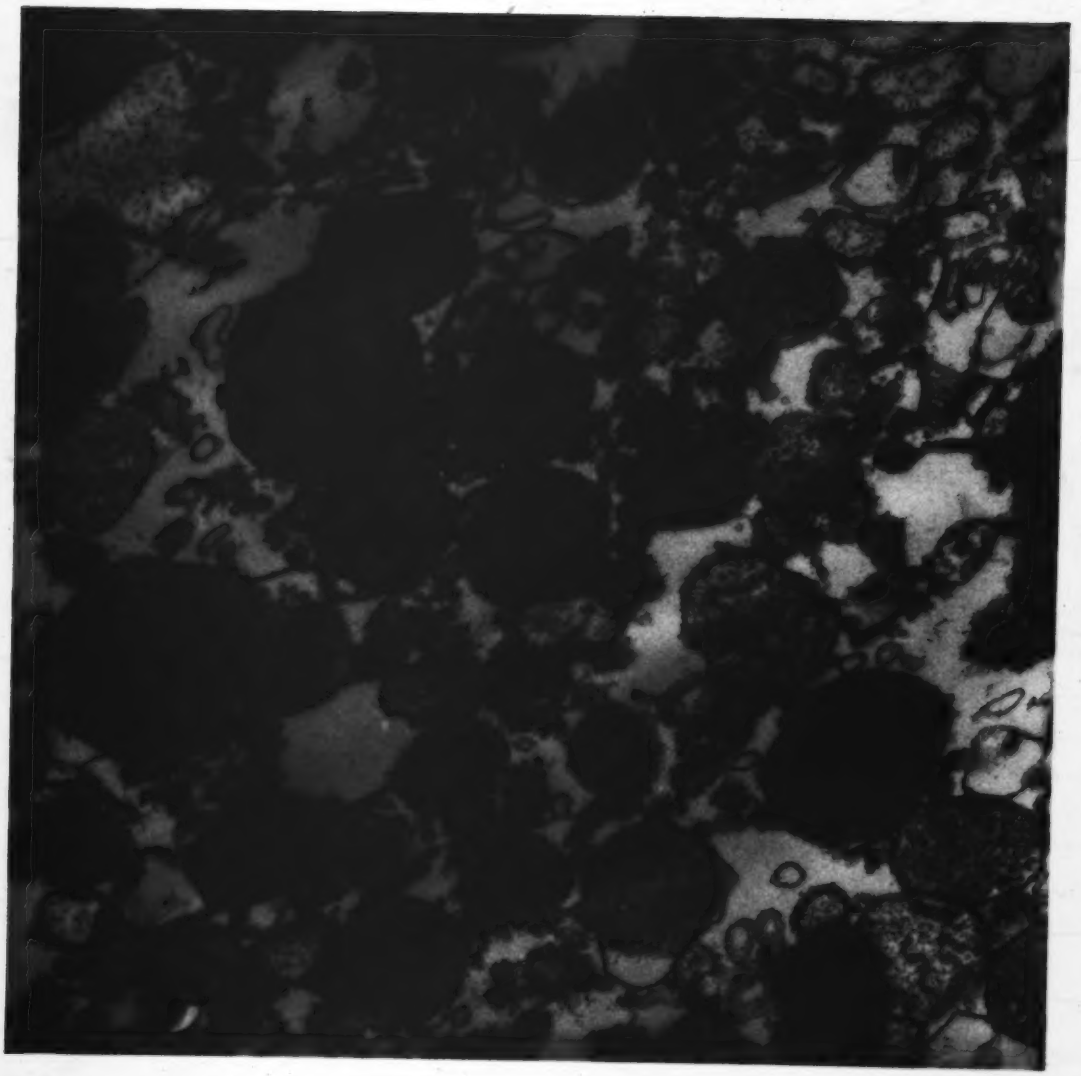


Plate 9 Negatively-stained preparation of liver subcellular membrane from a tocopherol-deficient rat. x 50,000

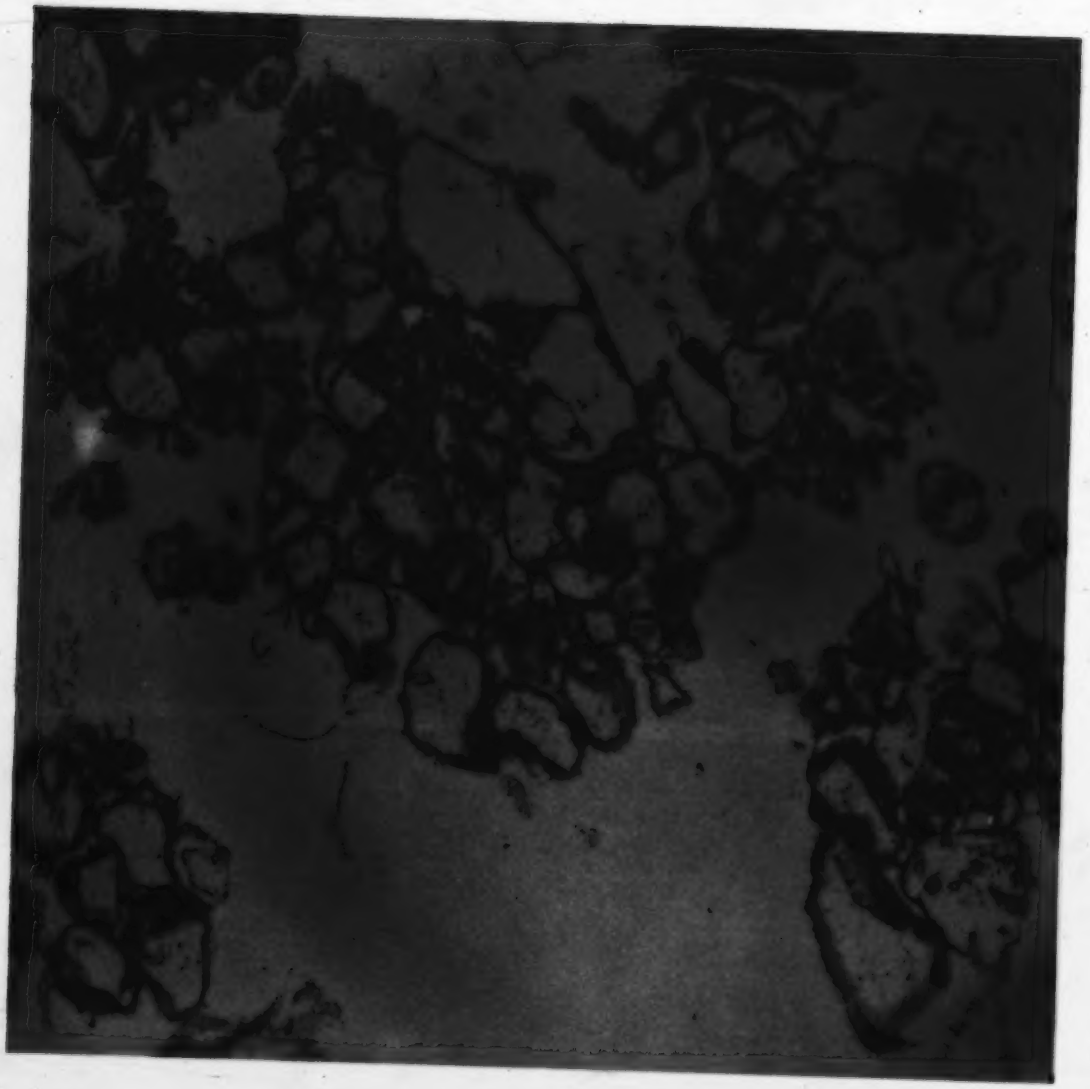
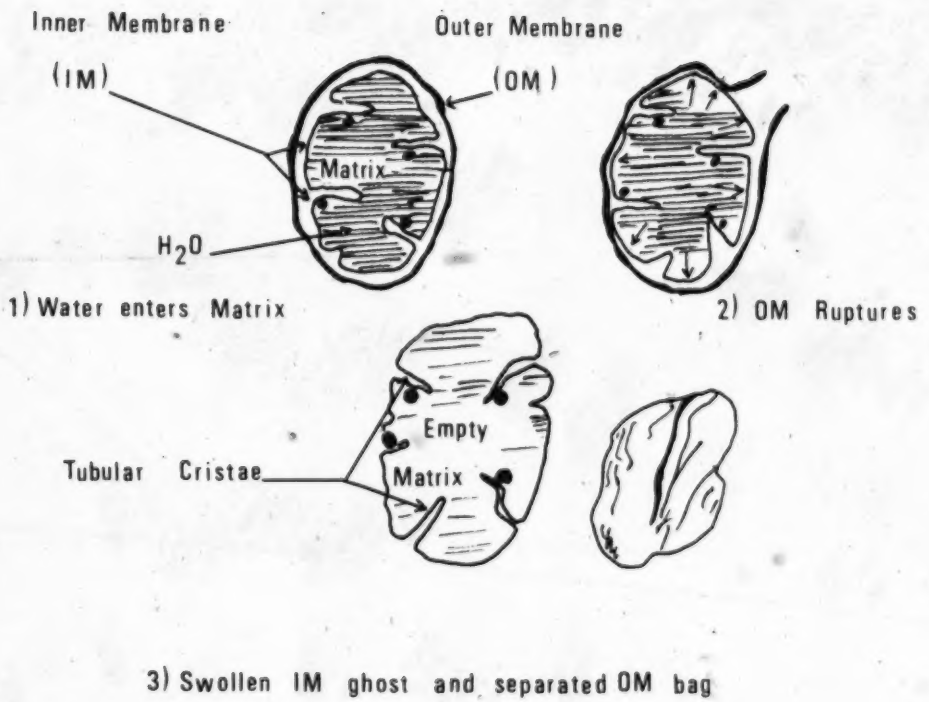


Figure 10. Effect of large amplitude swelling on outer and inner mitochondrial membrane (126).



F. Phospholipid Analysis

The results of the SM phospholipid analyses are presented in Tables 16 through 21. The data for testes tissue are presented independently of that for liver tissue. The data were calculated (see Appendix XII) using a phosphorus standard curve prepared according to linear regression analysis to find the line of best points.

Analysis of the results showed that there was no statistical difference in the mean levels of the liver or testes SM phospholipids between the 2 groups. This finding was evident when the data was expressed in a variety of ways, including expression of SM phospholipid levels per g whole tissue, per g of SM and per mg SM protein.

There is little data available on the effect of dietary vitamin E on cellular or SM phospholipids. Carpenter (26) has reported that the levels of rat testes total phospholipid decrease in response to vitamin E deficiency. The level of phospholipid was found to decrease as tissue degeneration occurred. The testes phospholipid levels of vitamin E-deficient rats were similar to those of controls until 3 mos of age. At 4 mos the total testes phospholipid levels of experimental rats were 70% those of the control rats. This was attributed to membrane loss of germinal epitheluim and interstitial tissue. The change in testes total phospholipid levels was unaccompanied by any change in the distribution of phospholipids.

Bieri and Prival (12) also have reported that a significant loss (up to 40% after 28 wks) of phospholipid occurs in the testes tissue of vitamin E-deficient rats. They also found examples where on testis was small with a markedly damaged germinal epithelium, whereas the partner testis was normal. The decrease in phospholipid was not found to correlate with observed testicular degeneration (gross and histo-

Table 16. Effect of dietary vitamin E on levels of subcellular membrane phospholipid per gram rat liver tissue.

	PC[a]		PE[b]		DPG[c]		TOTAL[d]	
Number	C[e]	E[f]	C	E	C	E	C	E
8, 13	3.691	3.802	0.849	0.666	1.022	0.246	5.56	4.71
7, 15	5.355	5.093	3.911	2.389	1.076	1.024	10.34	8.51
4, 5	4.004	6.252	1.782	3.467	1.161	1.229	6.95	10.95
14, 18	4.832	3.684	2.757	2.603	2.266	0.841	9.86	7.13
1, 20	1.733	3.176	1.078	1.636	--*[g]	0.664	3.84	5.48
16, 17	1.795	3.238	0.686	0.299	--*	--*	3.51	4.47
3, 11	4.920	4.968	2.544	2.280	1.068	1.242	8.53	8.49
2, 12	4.512	4.272	1.368	2.280	0.804	1.524	6.68	8.08
10, 19	4.809	4.263	1.680	2.184	0.221	0.630	6.71	7.08
6, 9	2.646	3.150	1.722	1.071	0.630	1.023	5.00	5.24
MEAN	3.830	4.190 (NS)[i]	1.838	1.888 (NS)	1.031	0.936 (NS)	6.70	7.01 (NS)
SEM[h]	1.330	1.006	0.988	0.966	0.587	0.386	2.30	2.06

a. Values expressed in mg SM PC per g liver tissue.
b. Values expressed in mg SM PE per g liver tissue.
c. Values expressed in mg SM DPG per g liver tissue.
d. Values expressed in mg SM phospholipid per g liver tissue.
e. Indicates control group.
f. Indicates experimental group.
g. * indicates values not available (see Appendix XVII).
h. Indicates standard error of the mean.
i. NS Indicates no significant difference between the mean of the 2 groups.

Table 17. Effect of dietary vitamin E on levels of subcellular membrane phospholipid per gram rat testes tissue.

	PC[a]		PE[b]		DPG[c]		TOTAL[d]	
Number	C[e]	E[f]	C	E	C	E	C	E
8, 13	2.316	3.048	1.331	2.406	0.401	0.306	4.05	5.76
7, 15	3.604	2.732	2.515	0.951	0.698	0.412	6.82	4.10
4, 5	3.979	4.638	3.259	4.506	1.743	1.811	8.98	10.96
14, 18	3.453	3.853	1.763	2.958	1.038	1.314	6.25	8.13
1, 20	2.567	2.244	1.362	1.562	0.360	0.352	4.29	4.16
16, 17	1.299	1.525	0.480	1.373	--*[g]	--*	12.49	3.54
3, 11	1.813	1.518	1.085	1.011	0.357	0.175	3.26	2.70
2, 12	1.043	1.837	0.861	0.918	--*	0.459	2.62	3.21
10, 19	2.112	2.339	1.062	1.974	--*	0.556	3.89	4.87
6, 9	2.568	2.243	1.177	2.124	0.411	0.346	4.10	4.71
MEAN	2.475	2.598 (NS)[i]	1.490	1.978 (NS)	0.715	0.637 (NS)	4.68	5.21 (NS)
SEM[h]	0.974	1.010	0.827	1.116	0.517	0.549	2.10	2.50

a. Values expressed in mg SM PC per g testes tissue.
b. Values expressed in mg SM PE per g testes tissue.
c. Values expressed in mg SM DPG per g testes tissue.
d. Values expressed in SM phospholipid per g testes tissue.
e. Indicates control group.
f. Indicates experimental group.
g. * indicates values not available (see Appendix XVII).
h. Indicates standard error of the mean.
i. NS indicates no significant difference between the mean of the 2 groups.

Table 18. Effect of dietary vitamin E on levels of subcellular membrane phospholipid per gram rat liver subcellular membrane.

	PC[a]		PE[b]		DPG[c]		TOTAL[d]	
Number	C[e]	E[f]	C	E	C	E	C	E
8, 13	21.71	16.53	4.99	2.90	6.01	1.07	32.7	20.5
7, 15	17.27	31.83	12.62	14.93	3.47	6.40	33.4	53.2
4, 5	26.69	27.18	11.88	15.07	7.74	5.34	46.3	47.6
14, 18	34.51	21.67	19.69	15.31	16.19	4.95	70.1	41.9
1, 20	13.33	18.68	8.29	9.62	--*[g]	3.91	27.8	32.2
16, 17	8.98	24.91	3.43	2.30	--*	--*	18.6	31.8
3, 11	35.14	22.58	18.17	10.36	7.63	5.65	60.9	38.6
2, 12	17.35	20.34	5.26	10.86	3.09	7.26	25.7	38.5
10, 19	28.29	15.79	9.88	8.09	1.30	2.33	39.5	26.2
6, 9	16.54	13.70	10.76	4.66	3.94	4.45	31.2	22.8
MEAN	21.98	21.32 (NS)[i]	10.50	9.41 (NS)	6.17	4.60 (NS)	38.6	35.2 (NS)
SEM[h]	8.88	5.57	5.40	4.92	4.64	1.90	16.2	10.6

a. Values expressed in mg SM PC per g liver SM.
b. Values expressed in mg SM PE per g liver SM.
c. Values expressed in mg SM DPG per g liver SM.
d. Values expressed in mg SM phospholipid per g liver SM.
e. Indicates control group.
f. Indicates experimental group.
g. * indicates values not available (see Appendix XVII).
h. Indicates standard error of the mean.
i. NS Indicates no signficant difference between the mean of the 2 groups.

Table 19. Effect of dietary vitamin E on levels of subcellular membrane phospholipid per gram rat testes subcellular membrane.

Number	PC[a]		PE[b]		DPG[c]		TOTAL[d]	
	C[e]	E[f]	C	E	C	E	C	E
8, 13	14.48	23.45	8.32	18.51	2.51	2.35	25.3	44.3
7, 15	21.20	17.08	14.79	5.94	4.11	2.58	40.1	25.6
4, 5	28.42	38.65	23.28	37.55	12.45	15.09	64.2	91.3
14, 18	21.58	22.66	11.02	17.40	6.49	7.73	39.1	47.8
1, 20	21.39	18.70	11.35	13.02	3.00	2.93	35.7	34.7
16, 17	11.81	9.53	4.36	8.58	--*[g]	--*	21.1	23.0
3, 11	12.95	15.18	7.75	10.11	2.55	1.75	23.3	27.0
2, 12	8.02	13.12	6.62	6.56	--*	3.28	19.6	23.0
10, 19	21.12	21.26	10.62	17.95	--*	5.05	36.7	44.3
6, 9	21.40	18.69	9.81	17.70	3.43	2.88	34.6	39.3
MEAN	18.24	19.83 (NS)[i]	10.79	15.33 (NS)	4.93	4.85 (NS)	34.0	40.0 (NS)
SEM[h]	6.14	7.89	5.24	9.21	3.59	4.25	13.1	20.3

a. Values expressed in mg SM PC per g testes SM.
b. Values expressed in mg SM PE per g testes SM.
c. Values expressed in me SM DPG per g testes SM.
d. Values expressed in mg SM phospholipid per g testes SM.
e. Indicates control group.
f. Indicates experimental group.
g. * indicates values not available (see Appendix XVII).
h. Indicates standard error of the mean.
i. NS Indicates no significant difference between the mean of the 2 groups.

Table 20. Effect of dietary vitamin E on levels of subcellular membrane phospholipid per milligram rat liver subcellular membrane protein.

Number	PC[a]		PE[b]		DPG[c]		TOTAL[d]	
	C[e]	E[f]	C	E	C	E	C	E
8, 13	0.710	0.704	0.163	0.123	0.197	0.046	1.07	0.87
7, 15	0.687	0.637	0.501	0.299	0.138	0.128	1.33	1.06
4, 5	0.755	0.665	0.336	0.369	0.219	0.131	1.31	1.17
14, 18	0.478	0.722	0.273	0.510	0.224	0.165	0.98	1.38
1, 20	0.299	0.599	0.186	0.309	--*[g]	0.125	0.64	1.03
16, 17	0.332	0.540	0.127	0.050	--*	--*	0.61	0.74
3, 11	0.507	0.497	0.262	0.228	0.110	0.124	0.88	0.85
2, 12	0.868	0.806	0.263	0.430	0.155	0.288	1.29	1.52
10, 19	0.943	0.789	0.329	0.404	0.043	0.117	1.32	1.31
6, 9	0.473	0.618	0.308	0.210	0.113	0.201	0.89	1.03
MEAN	0.605	0.658 (NS)[i]	0.275	0.293 (NS)	0.150	0.147 (NS)	1.03	1.10 (NS)
SEM[h]	0.220	0.100	0.110	0.140	0.060	0.070	0.28	0.25

a. Values expressed in mg SM PC per mg liver SM protein.
b. Values expressed in mg SM PE per mg liver SM protein.
c. Values expressed in mg SM DPG per mg liver SM protein.
d. Values expressed in mg SM phospholipid per mg liver SM protein.
e. Indicates control group.
f. Indicates experimental group.
g. * indicates values not available (see Appendix XVII).
h. Indicates standard error of the mean.
i. NS Indicates no significant difference between the mean of the 2 groups.

Table 21. Effect of dietary vitamin E on levels of subcellular membrane phospholipid per milligram rat testes subcellular membrane protein.

	PC[a]		PE[b]		DPG[c]		TOTAL[d]	
Number	C[e]	E[f]	C	E	C	E	C	E
8, 13	0.565	0.782	0.325	0.617	0.098	0.078	0.99	1.48
7, 15	0.680	0.515	0.475	0.179	0.132	0.078	1.29	0.77
4, 5	0.485	0.483	0.397	0.469	0.213	0.189	1.10	1.14
14, 18	0.432	0.688	0.220	0.528	0.130	0.235	0.78	1.45
1, 20	0.242	0.374	0.128	0.260	0.034	0.059	0.40	0.69
16, 17	0.157	0.175	0.058	0.158	--*[g]	--*	0.32	0.44
3, 11	0.218	0.271	0.131	0.181	0.043	0.031	0.39	0.48
2, 12	0.183	0.656	0.151	0.328	--*	0.164	0.44	1.15
10, 19	0.346	0.371	0.174	0.313	--*	0.088	0.62	0.77
6, 9	0.414	0.380	0.190	0.360	0.066	0.059	0.67	0.80
MEAN	0.372	0.470 (NS)[i]	0.225	0.339 (NS)	0.102	0.109 (NS)	0.70	0.92 (NS)
SEM[h]	0.170	0.190	0.130	0.160	0.060	0.070	0.33	0.37

a. Values expressed in mg SM PC per mg testes SM protein.
b. Values expressed in mg SM PE per mg testes SM protein.
c. Values expressed in mg SM DPG per mg testes SM protein.
d. Values expressed in mg SM phospholipid per mg testes SM protein.
e. Indicates control group.
f. Indicates experimental group.
g. * indicates values not available (see Appendix XVII).
h. Indicates standard error of the mean.
i. NS Indicates no significant difference between the mean of the 2 groups.

logical).

Donovan and Menzel (43) found that vitamin E had no significant effect on mouse lung total phospholipid, triacylglycerol, or individual fatty acid levels, either on lard or corn oil-based dietary regimens.

Grinna (60) investigated the effect of dietary vitamin E on rat liver mitochondrial and microsomal total phospholipids and found a slight[16], but not statistically significant, decrease in the mitochondrial total phospholipid levels of vitamin E-deficient rats as compared with control rats. There was no difference in microsomal phospholipid levels between the 2 groups. The sample size (3 membrane preparations of 2 pooled rats each) was so small as to preclude the likelihood of demonstrating a difference. Grinna also reported that there was no difference in growth or organ weight between the 2 groups.

The lack of observed difference in phospholipid levels in Grinna's study is consistent with my findings. However, this lack of difference might be due to microsomal contamination during the SM isolation process subsequent to tissue freezing.

The positive correlation between hemolysis and the loss of PE, which has been demonstrated in vitamin E-deficient RBC membrane (74, 81) may have reflected the in vitro design of those particular experiments. Such experiments do not necessarily demonstrate what happens to tissues under natural conditions, or even when the tissues are "naturally" exposed to oxidizing agents. Chemically, vitamin E is an antioxidant and acts in the same manner as many other chemical antioxidants (158, 159, 172). However, that vitamin E has some unique properties as a biological antioxidant is supported by the finding of several investigators that restoration of antioxidant activity by chemical antioxidants will

not reverse the biochemical lesion produced by the deficiency of vitamin E (25, 73). Upon in vitro exposure of the RBC membrane to powerful oxidizing agents such as dialuric acid or H_2O_2, one might expect these agents to have profound effects on the lipid component, such as a decrease in PE levels, if there were no chemical antioxidant present.

Tappel (158, 159) points out that there is a limit to the in vivo antioxidant protection offered by vitamin E because the molar ratio of membrane PUFA to vitamin E has been calculated to be 1000:1. In order to test the true in vivo antioxidant effect of vitamin E on membrane lipids one would have to subject the experimental and control animals to 'natural' environmental stresses prior to sacrifice. Such investigations have been conducted by Levander (94-97) who has intensively studied the effects of lead poisoning on the RBC membrane of vitamin E-deficient and control rats. There are many substances that can catalyze lipid peroxidation in biological systems, including molecular oxygen, ascorbic acid, iron and hemeproteins (37, 158, 159, 172) while environmental agents include ethanol, enrivonmental air pollutants (eg. ozone) and radiation (32, 43, 133, 159).

In Table 22 the levels of control SM total phospholipids determined in this study are compared with SM phospholipid levels determined by other investigators.

The values determined in this study are close to total phospholipid values for testes mitochondria reported by Krause et al (89), and are much higher than the mitochondrial phospholipid values reported by Grinna (60), Colbeau et al (34) and Rouser et al (139). When the phospholipid levels are expressed in mg total SM phospholipid per g tissue they are lower than the corrected values for mitochondrial membrane phospholipid reported by Ricquier et al (136), and are approxi-

Table 22. Comparison of control subcellular membrane phospholipid levels of the present study with the levels obtained by other investigators.

Investigator	Tissue	Membrane	Phospholipid Level[a]	b
This Study	rat liver	subcellular membrane	1.03	6.7
This Study	rat testes	subcellular membrane	0.70	4.7
Grinna (60)	rat liver	mitochondria	0.19	-
Grinna (60)=	rat liver	microsomes	0.37	-
Krause et al (89)	rat testes	total testes	-	13.4[c]
Krause et al (89)	rat testes	mitochondria	0.85	-
Krause et al (89)	rat testes	microsomes	0.42	-
Colbeau (34)	rat liver	mitochondria	0.35	-
Colbeau (34)	rat liver	microsomes	0.35	-
Ricquier et al (136)	rat brown adipose	mitochondria	-	8.4*[d]
Rouser (139)	beef liver	mitochondria	0.18	-
Rouser (139)	beef liver	rough microsomes	0.82	-
Rouser (139)	beef liver	smooth microsomes	1.15	-

a. Values are expressed in mg SM total phospholipid per mg SM protein.
b. Values are expressed in mg SM total phospholipid per g tissue.
c. Value is expressed as mg total tissue phospholipid per g testes tissue.
d. * indicates value is corrected to theoretical yield based on enzyme assay. The experimental value was reported to be lower.

mately 43% of the value for total testes membrane phospholipid reported by Krause et al (89). Carpenter (26) has reported similar (14.2 - 14.6 mg phospholipid per g testis tissue) values as those of Krause for total testes phospholipid levels.

The wide range of values for SM phospholipid reflect the difficulty in obtaining pure membrane preparations. Some investigators (89) report that mitochondria have more phospholipid than microsomes, while others (60, 139) claim the reverse. Colbeau (34) reports that the levels are similar. Since the majority of data reported by other investigators presents the quantity of total mitochondrial phospholipid as being lower than the values of the present experiment, it is likely that the SM fraction, while comprising a large percentage of mitochondrial membrane, also included a significant percentage of microsomal membrane, and possibly other SM (such as ER). This likely was a result of the tissue being frozen prior to SM isolation, and the use of sonication in the experimental procedure (126).

The relative contribution of the 3 phospholipids in percentage is expressed in Tables 23 and 24. Since an important criteria for establishing membrane purity is in the relative contribution of the various phospholipids, the percentage composition of control SM phospholipids obtained in my study is compared with the data of others in Table 25. It is evident that the presence of significant amounts of DPG indicate the presence of a significant amount of mitochondrial membrane, particularly IM. The latter claim was also suggested from the appearance of the membranes in the negatively-stained electron microscopic preparations. The percentage contribution of PE was very similar to the percentage of mitochondrial PE reported by others. The percentage contribution of PC found in this study was lower than the level for micro-

Table 23. Effect of dietary vitamin E on the percentage composition of rat liver phospholipids.

Number	PC[a]		PE		DPG	
	C[b]	E[c]	C	E	C	E
8, 13	66	81	15	14	18	5
7, 15	52	60	38	28	10	12
4, 5	58	57	26	32	17	11
14, 18	49	52	28	37	23	12
1, 20	47	58	29	30	23*[d]	12
16, 17	54	73	21	7	25*	20*
3, 11	58	58	30	27	13	15
2, 12	67	53	20	28	12	19
10, 19	71	60	25	31	3	9
6, 9	53	60	35	20	13	20
MEAN	58	61	27	25	15	14
SEM[e]	8.1	9.0	6.9	9.1	6.8	5.0

a. Values for all phospholipids are rounded to nearest %.
b. Indicates control group.
c. Indicates experimental group.
d. * indicates mean value for group has been used in the calculations.
e. Indicates standard error of the mean.

Table 24. Effect of dietary vitamin E on the percentage composition of rat testes phospholipids.

	PC[a]		PE		DPG	
Number	C[b]	E[c]	C	E	C	E
8, 13	57	53	33	42	10	5
7, 15	53	67	37	23	10	10
4, 5	44	42	36	41	19	17
14, 18	55	47	28	36	17	16
1, 20	61	54	32	38	9	9
16, 17	49	40	18	36	32*[d]	25*
3, 11	56	56	33	38	11	6
2, 12	42	57	34	29	23*	14
10, 19	56	48	28	41	16*	11
6, 9	62	48	28	45	10	7
MEAN	54	51	31	37	15	12
SEM[e]	6.7	7.9	5.5	6.5	7.4	6.1

a. Values for all phospholipids are rounded to nearest %.
b. Indicates control group.
c. Indicates experimental group.
d. * indicates mean value for group has been used in the calculations.
e. Indicates standard error of the mean.

Table 25. Comparison of the percentage composition of control subcellular membrane phospholipids of the present study and those obtained by other investigators.

Investigator	Tissue	Membrane	PC	PE	DPG
This Study	rat liver	subcellular membrane	58	27	15
This Study	rat testes	subcellular membrane	54	31	15
Bidlack et al (7)	rat liver	microsomes	67	22	-*[a]
Colbeau et al (34)	rat liver	mitochondria	41	35[b]	15*
Colbeau et al (34)	rat liver	microsomes	59	26[b]	2*
Getz et al (57)	rat liver	mitochondria	43	23	14*
Getz et al (57)	rat liver	microsomes	58	11	1*
Ricquier et al (136)	rat brown adipose	mitochondria	43	28	17*
Rouser (139)	guineau pig liver	mitochondria	40	28	23*
Rouser (139)	guineau pig liver	microsomes	63	18	1*
	MEAN[c]	mitochondria	42	29	17
	MEAN[c]	microsomes	62	19	1

a. * indicates percentages do not total 100 because other minor phospholipids were quantitated but are not reported.

b. Reported to include phosphatidyl serine which represented 0.9% total lipid phosphorus.

c. Represents average of percentages reported by other investigators (7, 34, 57, 136, 139).

somal PC and higher than the level for mitochondrial PC found by other investigators (see Table 25). The fact that between 5 and 10% of the total phospholipids were not quantitated in the present experiment must be taken into account, but it would not alter the relative contribution of the 3 major phospholipids to a significant degree.

The high values for standard deviation are an important factor in the interpretation of the results of the present study. There was a greater degree of variation in the values for PE and DPG than for PC, likely a result of more degradation of those 2 phospholipids in some samples. With a few exceptions (see Appendix XVII) the reproducability of results within the triplicate determinations of each sample was good. The large range of values occurred between different samples. The extremely low values reported for some samples cannot be explained on the basis of prolonged storage, since some low values were found among the first samples to be analyzed. (The data is presented in the order that the SM fractions were reisolated and analyzed for phospholipids.) The effect of the prolonged storage must still be considered as a factor. The extremely sensitive nature of PE and DPG must also be considered. Rouser et al (139), when discussing the loss of PE which may occur when lipid extracts are evaporated to dryness, reports that when BHT is added to the extraction solvent, prolonged storage of the extract is possible. This was found to be true in several samples when the initial lipid extracts were chromatographed and quantitated, however, the DPG component had completely decomposed in spite of the rigorous precautions. Also, the level of PE in several of the original samples was extremely low. The lower standard deviations for PC reflected the more stable nature of that phospholipid. When investigating the phospholipid composition of guinea-pig liver microsomes and mitochondria, Rouser et al report

that many analyses were invalidated because both PE and DPG had decomposed upon standing prior to analysis. While this fact was considered in the interpretation of the present results, it was decided to use all of the results, because it is impossible to identify which values reflect low tissue values, and which values reflect analytical mishandling. The only values removed were those in which the original interpolated value for phosphorus concentration was less than 1 μg, which was the level of sensitivity chosen for the method (see Appendix XVII). All other 'low' values were included in the statistical analyses with the exception of 5 values which are explained in Appendix XVII.

An interesting consideration of the present data is the slightly higher mean values of SM PE in the vitamin E-deficient group, when compared with the control group. The mean values of testes SM PE for the control group range from 66% to 75% of the values for the experimental group, depending on the manner in which the data are expressed. While the differences between the means are noticeable, the high standard deviations removes any statistical significance. This finding is opposite to that observed in vitamin E-deficient hemolyzed RBC's (74, 81). It is similar to the finding of Krause et al (89), who investigated the effect of dietary retinol on rat testes mitochondrial and microsomal phospholipids. These investigators found that there was a significant increase in both the level and turnover of mitochondrial phospholipids of the retinol-deficient rat testes tissue. There was no difference between the 2 groups in the levels of microsomal phospholipids, a finding similar to that of Grinna. Also interesting was the finding that the phospholipid levels of liver mitochondria were unaffected by dietary manipulation of retinol. The authors speculate that the difference is related to some manner of cellular degeneration observed

in testes, and not liver tissue. While these findings may seem unrelated to those of this experiment, it is important to note that there is an interrelationship between vitamin E and A (50, 59, 82, 84, 154). Administration of vitamin E has been shown to significantly increase plasma vitamin A levels in both normal children and in those with vitamin A deficiency (82). It has been suggested that vitamin E may improve the absorption of vitamin A, increase the mobilization of vitamin A from the liver, or reduce oxidative destruction of vitamin A in circulation (82).

The difference in tissue response to retinol deficiency reported by Krause has been observed by investigators studying the effect of vitamin E deficiency on tissue total lipid fatty acid levels. Lee and Barnes (91) report that in testes, muscle and brain tissue the percentage of total lipid PUFA is decreased in response to vitamin E deficiency, while in lung, pancreas, heart and kidney, the level of PUFA increases, and in liver, spleen and small intestine there was no consistent pattern of change observed. These investigators report that the tissue exhibiting the most significant change was testes tissue. Farnsworth et al (49) found that vitamin E deficiency was associated with a large decrease in the total PUFA of rat retinal pigment epitheluim, while in the retinal rod outer segments only a small decrease was produced, and no change was produced in whole retina or liver tissue. Such differences in tissue response may explain why Donovan and Menzel (43) found no association between dietary vitamin E and mouse lung lipids.

The slight increase in the level of testes SM PE which was observed in the experimental group occurred without a decrease in the level of PC. In fact, the level of PC and total phospholipid also were slightly higher (see Table 21). When evaluating the level of any one

phospholipid it is important to note whether a shift in the percentage contribution of other phospholipids has occurred. For example, it might be possible for a shift from PE to PC (correspondingly increasing the saturation of the fatty acids) to occur as a first line of defense against vitamin E deficiency. Such changes have been demonstrated in the brown adipose tissue mitochondria of rats exposed to cold environments, where the level of PE increases and PC decreases (136). Obviously, this did not occur in the present experiment.

Another important factor in the interpretation of the present data concerns the recovery of the phospholipids following the extraction of the SM after freezing (see Appendix XII). The levels of phospholipids were compared with those obtained from a freshly killed rat. The separation and quantitation of the phospholipids was conducted with samples 4, 5 and 14, 18. When compared with the levels from the freshly killed (reference) rat, the levels of liver SM phospholipids for control rats 4, 5 and 14, 18 averaged 82%. For testes SM the recovery was 80%. When the phospholipid levels of all the samples were compared with those of the reference rat, the recovery percentage was much lower, reflecting the large variation in values. This large variation in values might also have been produced if a greater number of reference rats had been used to compare the recovery. I would have expected the values for the reference rat to have been much lower, since a more pure preparation of SM would have been expected from non-frozen tissue. This may reflect a limitation of the SM isolation method chosen.

The data presented in the present experiment has provided increased evidence that the manifestations of vitamin E deficiency are varied, and reflect the tissue and membrane types studied, as well as the age at which the deficiency state is produced.

In summary, the purpose of this experiment was to determine whether vitamin E affords any in vivo antioxidant protection to the highly susceptible (peroxidizable) subcellular membrane phospholipids of rat liver and testes. Subcellular membrane phopholipids, particularly those of mitochondria, are chiefly composed of PUFA, particularly arachidonic acid, which has been shown in rabbit muscle and in rat RBC to be very sensitive to vitamin E deficiency. Twenty weanling rats were fed a vitamin E-deficient powdered diet for 25 wks, and 20 control rats received an identical diet except for the addition of vitamin E. On the basis of serum and tissue vitamin E determinations, the experimental group was judged to be vitamin E-deficient. In retrospect, a number of factors must be considered which could have affected the results of this study, and they are summarized here.

The magnitude of the deficiency was questioned. Analysis of the experimental diet should have been conducted to rule out the possibility of vitamin E contamination. Also, the weanling rats were somewhat older and heavier than was anticipated and perhaps should have been rejected as subjects since their initial vitamin E stores could have significantly altered the results. Base-line serum and tissue alpha-tocopherol levels would have settled this doubt. Technical difficulties over laboratory methods, which led to the postponement of phospholipid analyses, caused a deterioration of the original lipid extract. These technical difficulties should have been overcome prior to initiating the animal feeding trial, in order to avoid the subsequent use of frozen tissue for the subcellular membrane isolation. The use of frozen tissue may have caused the large standard deviations, which made statistical comparisons difficult. In neither rat liver nor testes

subcellular membrane was there a statistically significant difference between the control and vitamin E-deficient groups in the levels of PC (phosphatidyl choline), PE (phosphatidyl ethanolamine) or DPG (diphosphatidyl glycerol). However, contrary to the initial hypothesis, but similar to that observed with retinol deficiency, the subcellular membrane PE, PC and total phospholipid levels appeared to be slightly increased in the vitamin E deficient rat testes tissue, and not in the liver tissue. The low tissue vitamin E levels, at least under the deficiency conditions produced in this experiment, did not appear to increase the rate of in vivo subcellular membrane phospholipid peroxidation. Vitamin E remains a vitamin still in search of a completely defined role in biological systems.

FOOTNOTES

1. Intake = food consumed in the previous 24 hrs.
2. Indicated by presence of blood in and around the nostrils.
3. Hematocrit values, reticulocyte count, spleen weight, RBC mechanical fragility and filterability of RBC's.
4. Twenty-two percent as calories, which corresponds to the level of corn oil used in the present investigation.
5. Total iron-binding capacity.
6. Rats were fed ad libitum with a diet identical to that used in the present experiment.
7. Fed 0.5 mg d-alpha-tocopherol acetate daily for 2 wks post weaning to elevate tissue stores.
8. Rats weighing 410 g to 440 g fed stock diet from weaning on.
9. As determined by in vitro incorporation of 1-^{14}C acetate into citrate.
10. Since mitochondria in general show little or no organ or species variability in composition (139), it does seem appropriate to compare the results even though the tissues in question are different.
11. Guerra (63) p. 319.
12. Guerra (63) p. 321.
13. Personal communication: Dr. J. P. Tewari, Department of Plant Science, University of Alberta.
14. Expressed as ng. atoms O/mg protein/min at 37° C using 8.3 mM glutamate/malate as substrate.
15. The samples are fixed in a medium of glutaraldehyde, post fixed in OsO_4 and sections were cut on an ultramicrotome.
16. 0.19 mg phospholipid per mg protein for the control group versus 0.15 mg phospholipid per mg protein for the experimental group.

VI SUMMARY

1. Prior to initiation of the feeding trial all weanling rats appeared to be in good health. The mean weight for the control and experimental group was 81 and 82 g respectively, while the mean age of the control group was 27 days versus 26 days for the experimental group. Distribution of litter mates between the 2 groups was similar.

2. The mean food intake of the 2 groups was similar throughout the feeding trial.

3. The growth curves of both groups were similar throughout the feeding trial. At weeks 19 and 20 the mean weight of the experimental group was significantly lower than that of the control group but this difference disappeared on week 21, and for the remainder of the feeding trial there was no significant difference in mean weight between the 2 groups.

4. Between weeks 19 and 20 the experimental group developed a diarrheal illness, and within 2 to 3 days the control rats also were afflicted. All rats appeared to have recovered from the illness by the end of week 20. With the exception of a brief weight-lowering effect, which affected all rats in a similar manner, the diarrheal episode did not appear to significantly affect the outcome of the experiment.

5. The rats were sacrificed over a 4 week period beginning at the 24th week of the feeding trial. A comparison of the physical signs present in the control versus experimental groups at the conclusion of the experiment revealed that a significantly greater number of rats from the experimental group had nasal porphyria, a common sign of vitamin E deficiency. However, the difference in the incidence of greasy-rough coat, another sign of vitamin E deficiency, was not statistically significant.

There was no evidence of other vitamin E deficiency signs such as kyphosioliosis, tremors or skin ulcerations present in any of the rats.

6. Hematocrit levels in both the experimental and control groups were similar, indicating that this parameter was not affected by dietary vitamin E.

7. The mean weights for liver and testes tissue between the 2 groups were unaffected by dietary vitamin E.

8. There was a statistically significant difference in the mean value for serum alpha-tocopherol between the 2 groups, with the mean value for the control group being approximately 10 times greater (1.41 mg % versus 0.16 mg %) than that of the experimental group. According to this parameter the experimental group was judged to be vitamin E-deficient.

9. There was a statistically significant difference in tissue alpha-tocopherol levels between the 2 groups, although the magnitude of this difference was less than that observed for serum alpha-tocopherol. The values for liver alpha-tocopherol were 31.4 μg/g for the control group and 10.1 μg/g for the experimental group while the values for testes alpha-tocopherol were 22.1 μg/g and 6.9 μg/g respectively. These levels were not as low as those reported by other investigators, but they were sufficiently low to conclude that the experimental group was vitamin E-deficient.

10. Evaluation of the SM pellet weight of the 2 groups revealed that dietary vitamin E had no effect on the amount of SM. There were no differences between the 2 groups in the amount of liver SM protein. Analyses of testes SM protein showed no difference between the 2 groups.

11. Electron microscopic examination of negatively-stained preparations of liver and testes tissue SM from control and experimental rats showed no differences in SM ultrastructure, although some photographs showed

vitamin E-deficient mitochondria with loss of matrix.

12. The data for liver SM phospholipid levels was expressed in a number of different ways, SM phospholipid per g whole tissue, per g of SM, and per mg SM protein, but no way demonstrated a significant difference between the control and experimental groups. A comparison of testes SM phospholipid levels between the control and experimental groups revealed that there were no significant differences in the mean levels of PC or DPG. While the mean values of PE for the experimental group ranged from 1.3 to 1.5 times higher than those of the control group the differences were not judged to be significant. The large values for standard deviation may have masked any true difference.

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APPENDIX I

Composition of Salt Mixture No. 4164

Calcium Carbonate	16.356%
Cupric Sulfate - $5H_2O$	0.018%
Dicalcium Phosphate - $2H_2O$	35.556%
Ferric Citrate - $3H_2O$	1.600%
Magnesium Carbonate	4.089%
Manganese Sulfate - H_2O	0.138%
Potassium Citrate - H_2O	23.653%
Potassium Iodide	0.004%
Potassium Phosphate Dibasic	7.733%
Socium Chloride	10.809%
Zinc Carbonate	0.044%

APPENDIX II

Vitamin Diet Fortification Mixutre

	g/kg food
Vitamin A Concentrate (200,000 I.U.'s/gram)	.099
Vitamin D Concentrate (400,000 I.U.'s/gram)	.006
Ascorbic Acid	.990
Inositol	.110
Choline Chloride	1.650
Menadione	.050
p-Aminobenzoic Acid	.110
Niacin	.099
Riboflavin	.022
Pyridoxine Hydrochloride	.022
Thiamine Hydrochloride	.022
Calcium Pantothenate	.066

	mg/kg food
Biotin	.440
Folic Acid	1.980
Vitamin B_{12}	.029

APPENDIX III

Standardized Procedure for Mixing Vitamin E into 5 Kilograms of Vitamin E-Deficient Powdered Diet.

1. Weigh 1.25 g dl-alpha-tocopherol acetate directly into a small mixing bowl which attaches to the weighing scale. Add 10 g of powdered food and using a spoon, mix manually for 30-45 sec until a homogeneous paste is formed.
2. Gradually mix 10 g spoonfuls of vitamin E-deficient powdered diet into the paste until a total of 100 g have been added.
3. Carefully transfer the mixture to a medium-sized (20 l) Hobart mixing bowl and add 900 g of powdered diet in three amounts of approximately 300 g each. Between each addition stir to mix using a laboratory spatula spoon.
4. Attach mixing bowl to an automatic Hobart food mixer and set machine at low speed (reading of 1). During first minute of mixing, gradually add 4 kg remaining vitamin E-deficient powdered diet. Leave mixing for 15 min.

APPENDIX IV

Composition of City of Edmonton Water

	Summer Months May, 1976 to Sept. 1976 Inclusive	Winter Months Oct., 1975 to April, 1976 Inclusive
Calcium Bicarbonate	38 p.p.m.	36 p.p.m.
Calcium Sulphate	20 p.p.m.	12 p.p.m.
Magnesium Sulphate	23 p.p.m.	33 p.p.m.
Magnesium Bicarbonate	0 p.p.m.	2 p.p.m.
Sodium Sulphate	17 p.p.m.	47 p.p.m.
Sodium Chloride	5 p.p.m.	5 p.p.m.
Organic Matter	9 p.p.m.	13 p.p.m.
Total Solids	112 p.p.m.	148 p.p.m.
Calcium	20.6 p.p.m.	18.09 p.p.m.
Magnesium	4.59 p.p.m.	7.07 p.p.m.
Sodium	7.49 p.p.m.	17.08 p.p.m.
Total Hardness	71.0 p.p.m.	77.0 p.p.m.
pH Value	9.1 p.p.m.	9.1 p.p.m.

APPENDIX V

Method for Microhematocrit Determination

1. Following the open cardiac puncture a considerable leakage of blood occurs. For each animal subject, fill two microhematocrit tubes 2/3 full of the blood pooled in the mediastinum around the heart.
2. Plug ends which have not been wet with a small amount of modeling clay.
3. Place tubes in appropriately labelled sections of a hematocrit rack.
4. Place tubes in order into slots of microcentrifuge head. Make certain that the plugged end is placed on the outer circumference of the head.
5. Centrifuge for 5 min.
6. Remove each tube and measure the percent packed cells using the microhematocrit reader.
7. Average the two readings.

APPENDIX VI

Method for Isolation of Subcellular Membrane Pellet

Liver Tissue

1. Weigh out 1 g[1,2] of blotted ice-cold liver tissue and place into a 20 ml pyrex tube-shaped Potter-Elvejhem pestle.
2. Add ice-cold isotonic (0.25 M) sucrose[3] to a volume of 10 ml (1.0 g liver tissue per 10 ml homogenate).
3. Place the mortar filled with tissue and isotonic sucrose into a beaker full of ice and homogenize continuously by hand for 1 min. This time period was found to be satisfactory for complete homogenization.
4. Pour homogenate through a layer of sterile gauze[4] into a labelled 15 ml centrifuge tube with a conical head. Centrifuge[5] under refrigeration for 10 min at 600 g to sediment the nuclei.
5. Pour supernatant[6] into a 10 ml plastic round bottom centrifuge tube and centrifuge[7] under refrigeration for 20 min at 8500 g to sediment the mitochondria.
6. Discard supernatant. Resuspend SM pellet[8] in 2.5 ml of ice-cold isotonic sucrose and sonicate[9] (lowest setting) for 5 sec. Centrifuge under refrigeration for 10 min at 8500 g. Repeat[10] 3 times using ice-cold isotonic sucrose, then repeat 2 times using ice-cold distilled water[11].
7. Resuspend final SM pellet with distilled water to a final volume of 4 ml.

Testes Tissue

1. Randomly select 1 testis from each of the 2 animals to be pooled (ie. a total of 2 testes)[12] and weigh together after the outer capsule has been removed.

2. Place both[13] decapsulated testes into a 20 ml pyrex tube-shaped Potter-Elvejhem pestle. Add ice-cold isotonic (0.25 M) sucrose to a volume of 10 ml (usually about 4.0 g testes tissue in 10 ml homogenate)[14].

3. Place the pestle filled with tissue and isotonic sucrose into a beaker full of ice and homogenize continuously by hand for 1 min. (This time period was found to be satisfactory for complete homogenization.) Pour homogenate into a 10 ml graduated cylinder. Pour 5 ml back into Potter-Elvejhem pestle and add remaining ice-cold isotonic sucrose to make final concentration 1g testes tissue to 10 ml homogenate. Homogenize continuously for about 30 sec. Pour into 50 ml pyrex flask and place flask in ice. Repeat process with remaining 5 ml so that the flask contains approximately 4 g testes tissue in 40 ml 0.25 M sucrose.[15]

4. Pour homogenate through a layer of sterile gauze into 3 labelled 15 ml conical head centrifuge tubes so that the testes homogenate is evenly distributed between the 3 tubes[16]. Each tube now is treated as 1 sample. Centrifuge under refrigeration for 10 min at 600 g to sediment the nuclei.

5. Pour supernatant[17] into a 10 ml plastic round bottom centrifuge tube and centrifuge under refrigeration for 20 min at 8500 g to sediment the mitochondria.

6. Discard supernatant. Resuspend SM pellet[18] in 1.25 ml of ice-cold 0.154 KCl[19] and sonicate[20] (lowest setting) for 5 sec. Combine contents of 2 tubes for a total volume of 2.50 ml[21]. Centrifuge under refrigeration for 10 min at 8500 g. Discard supernatant. Resuspend SM pellet in 2.5 ml of ice-cold 0.154 M KCl and sonicate (lowest setting) for 5 sec. Centrifuge under refrigeration for 10 min at 8500 g. Repeat 2 times[22] using ice-cold 0.154 M KCl, then repeat 2 times using ice-cold distilled water[23].

7. Resuspend final SM pellet with distilled water to a final volume of 4 ml.

FOOTNOTES

1. Approximately 0.5 g was used from each animal.
2. For the second run 0.5 g was used from each animal (see Figure 4).
3. Isotonic sucrose is used to prevent swelling or shrinking of the subcellular organelles during the isolation procedure, and to prevent any effect of swelling or shrinking on the sedimentation rate.
4. This step ensures removal of all connective tissue strands.
5. PR-6 Centrifuge, International Equipment Co. maintained at 5°C.
6. The SM pellet is assumed to be predominantly mitochondrial membrane.
7. Sorvall RC-2 Centrifuge.
8. The initial pellet of nuclear membranes is discarded.
9. Sonication was found to be necessary to ensure complete resuspension of the pellet.
10. The SM pellet is washed repeatedly to increase the concentration of mitochondria by removing microsomes and ER.
11. When mitochondria are exposed to sucrose, the intracristal space expands and the maxtrix space becomes condensed, thus the mitochondrial membranes are not evident in electron microscopy. Once the pellet has been purified with washings of 0.25 M sucrose, the distilled water is used to remove traces of the sucrose (165).
12. A modification was made for the second run so that only 1 randomly selected testis was used from the 2 animals to be pooled (see Figure 5).
13. For the second run this refers to only 1 decapsulated testis.
14. For the second run this usually was 1.5 to 2.0 g testes tissue in 10 ml homogenate.
15. For the second run this amount is reduced to approximately 2.0 g testes tissue in 20 ml 0.25 M sucrose.
16. For the first run this came to 12-13 ml per tube and for the second run 2 tubes were used and were filled approximately 10 ml.
17. The initial pellet of nuclear membranes is discarded.

Footnotes Continued

18. The SM pellet is assumed to be predominantly mitochondrial membrane.

19. 0.154 M KCl serves the same purpose as 0.25 M sucrose. By suspending the subcellular organelles in a solution of the same tonicity, any swelling or shrinking of the organelles is prevented, and therefore any effect of swelling or shrinking on the sedimentation rate is prevented.

20. Sonication was found to be necessary to ensure complete resuspension of the pellet.

21. The testes pellet weight was about half the size as that of the liver tissue, so 2 were combined to make 1 pellet of approximately the same size as the liver pellet. Thus, 2 pellets were obtained from approximately 4.0 g testes tissue for the first run and 1 pellet was obtained from 2.0 g testes tissue in the second run.

22. The SM pellet is washed repeatedly to increase the concentration of mitochondria by removing microsomes and ER.

23. As is the case with sucrose, when mitochondria are exposed to isotonic salt solutions, the intracristal space expands and the matrix space becomes condensed, thus the mitochondrial membranes are not evident in electron microscopy. Once the pellet has been purified with washings of 0.25 M sucrose, the distilled water is used to remove traces of the salt solution (165).

APPENDIX VII

Method for Determination of Alpha-Tocopherol Status

A. Preparation of Reagents

1. 0.12 gm% alpha, alpha[1]-dipyridyl in absolute ethanol. Weigh out 0.12 g alpha, alpha[1]-dipyridyl. Place in pyrex flask or beaker and add 100% ethanol to 100 ml. Mix thoroughly.

2. 0.12 gm% ferric chloride in absolute ethanol. Weigh out 0.12 g ferric chloride. Place in foil wrapped flask or beaker and add 100% ethanol to 100 ml. Cover. Mix thoroughly. Store in a foil wrapped air tight bottle or a brown glass bottle.

B. Preparation of Standard Solutions

Alpha-Tocopherol

NB. Because of the light sensitive behavior of vitamin E, all analytical procedures involving alpha-tocopherol standards or samples were conducted with the artificial room lighting turned off and the window blinds closed.

1. Weight out 2.0 mg dl-alpha-tocopherol acetate and place into labelled foil wrapped pyrex flask. Add 100% ethanol to 100 ml. Cover. Mix thoroughly. Store in the refrigerator in a tightly capped, foil wrapped brown bottle. Before capping, seal under a flow of nitrogen.

2. For each day of analysis prepare a fresh set of standard solutions according to the following table. Reseal stock solution with a flow of nitrogen. Place in refrigerator.

Tube #	Concentration in µg/.75 mls	mls stock alpha-tocopherol	mls 100% ethanol
1	0.00	0.0	4.0
2	3.75	1.0	3.0
3	7.50	2.0	2.0
4	11.25	3.0	1.0
5	15.00	4.0	0.0

3. From the standard solutions on the previous page prepare three aliquots of 0.75 ml and treat as for samples.

Beta-Carotene

1. Carefully weigh out 0.4 mg of beta-carotene. Place into foil wrapped pyrex beaker. Add xylene to 100 ml. Cover. Mix well. Pour into labelled foil wrapped stock bottle. Seal with nitrogen. Store in refrigerator.

2. For each day of analysis prepare a fresh set of standard solutions according to the following table. Reseal stock solution with a flow of nitrogen. Place in refrigerator.

Tube #	Concentration in µg/.75 mls	mls stock beta-carotene	mls xylene
1	0.00	0.0	4.0
2	0.75	1.0	3.0
3	1.50	2.0	2.0
4	2.25	3.0	1.0
5	3.00	4.0	0.0

3. From the above standard solutions, prepare three aliquots of 0.75 ml and treat as for samples.

C. Procedure for Serum Alpha-Tocopherol

1. Pipet 0.75 ml serum into a 100 x 15 mm pyrex test tube. Do in triplicate.

2. For each tube fill a 100 x 15 mm pyrex test tube with 0.75 ml distilled water (paired blank). Treat exactly as for serum.

3. Add 0.75 ml absolute ethanol to each tube. Mix thoroughly for 5 sec using a vortex mixer set at 1.

4. Add 0.75 ml xylene to each tube. Cap tube with cork cap. Mix for 2 min on a vortex mixer set at 1. Try to cause fairly violent agitation of the tubes contents while preventing too much liquid from spurting to the upper segment of the tube.

5. Centrifuge[1] under refrigeration[2] for 10 min at 3,000 r.p.m.'s.

6. Pipet 0.50 ml of the xylene supernatant layer to a clean 100 x 15 mm pyrex tube.

7. Add 0.50 ml 0.12 gm% alpha, alpha[1]-dipyridyl reagent. Mix for 5 sec on a vortex mixer set at 1.

8. Carefully decant contents of the tube into a Beckman microcuvette and read the absorption on a double-beam spectrophotometer set at 455 mμ.

9. Zero the instrument with a distilled water blank to which has been added all reagents. Read each tube containing serum or standard[3] against the blank. After reading the absorption at 455 mμ carefully decant the contents of each tube back into its 100 x 15 mm pyrex test tube.

10. Set spectrophotometer to 510 mμ.

11. Using one unknown tube (serum, tocopherol or carotene standard) and its' paired blank at a time add 0.25 ml 0.12 g% ferric chloride reagent. Time reaction for 90 seconds using a clock timer. Zero the instrument each time with the paired blank and read each tube against its own paired blank[4].

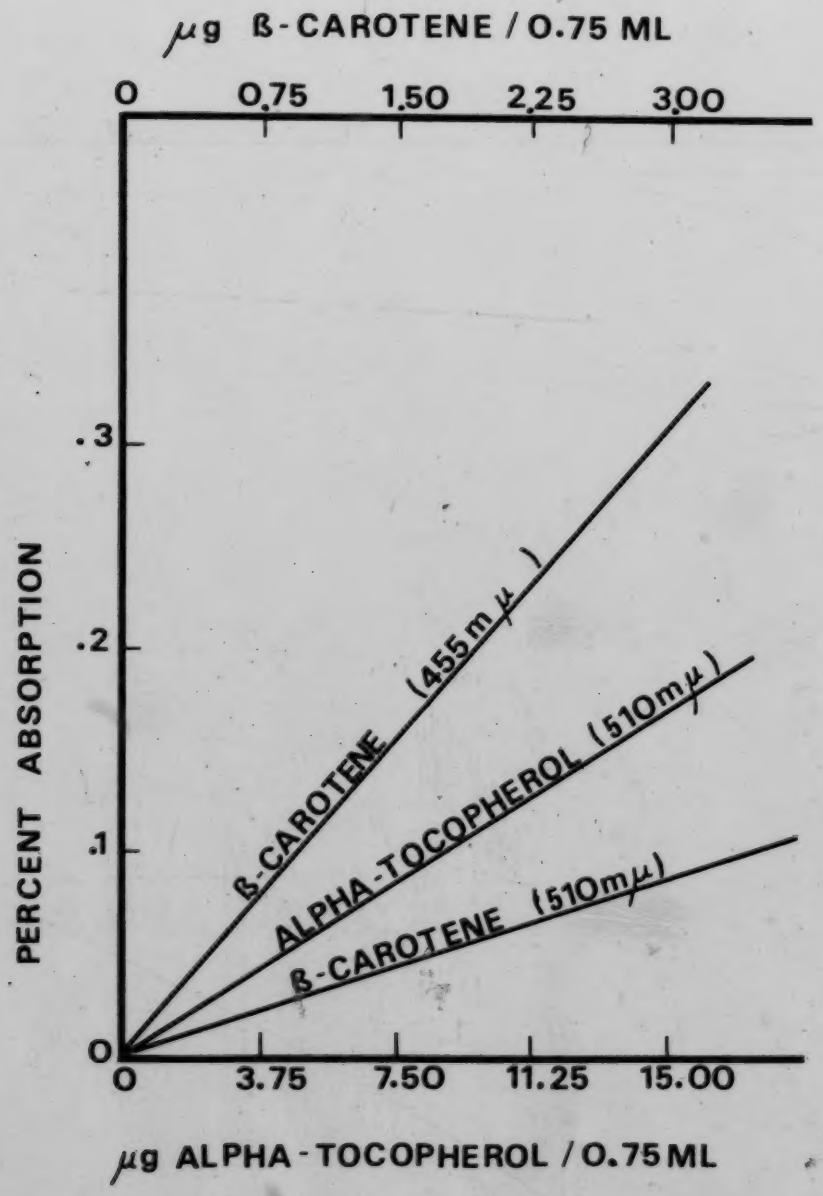
D. Calculations

1. Average the triplicate spectrophotometric readings (at 510 mμ) of the tubes containing alpha-tocopherol standard and plot against concentration of alpha-tocopherol (see Figure 11).

2. Average the triplicate spectrophotometric readings (at both 455 mμ and 510 mμ) of the tubes containing beta-carotene standard and plot against concentration of beta-carotene (see Figure 11).

3. Determine the ratio of beta-carotene at 510 mμ to beta-carotene at 455 mμ (eg. factor = .25).

Figure 11. Alpha-tocopherol and beta-carotene standard curves.



4. Determine the concentration of alpha-tocopherol according to the following formula.

$$\text{mg\% tocopherol} = \frac{D_{510} - .25^{5} D_{455}}{D_{510}\ \text{standard}} \times \text{mg\% standard}^{6}$$

D_{510} = the spectrophotometric reading (absorbance) of the sample at 510 mμ. D_{455} = the spectrophotometric reading (absorbance) of the sample at 455 mμ. D_{510} standard = see footnote 2.

5. Determine the concentration of beta-carotene by interpolating the actual concentration of beta-carotene in 0.75 ml with the spectrophotometric reading of the sample at 455 mμ. Multiply by a factor of 133, which simply expresses the beta-carotene concentration per 100 ml. This is possible because the standard solutions of beta-carotene are treated exactly as the samples.

E. Tissue Alpha-Tocopherol

1. Prepare a lipid extract of the liver and testis tissue according to a modification of the method by Folch (see Appendix IX).

2. For each sample, reduce volume of the lipid extract and bring to a volume of 2.25 ml in chloroform:methanol[7] (2:1).

3. Prepare three aliquots of 0.75 ml and treat as for serum alpha-tocopherol analysis using the same procedure for the beta-carotene and alpha-tocopherol standard solutions.

4. Determine the concentration of alpha-tocopherol according to the following formula.

$$\frac{\mu\text{g alpha-tocopherol}}{.75\ \text{ml}} = \frac{D_{510} - .25^{8} D_{455}}{D_{510}\ \text{standard}} \times \frac{\mu\text{g standard}^{9}}{.75\ \text{ml}}$$

D_{510} = the spectrophotometric reading (absorbance) of the sample at 510 mμ. D_{455} = the spectrophotometric reading absorbance) of the sample at 455 mμ. D_{510} = see footnote 2.

This value gives the concentration of alpha-tocopherol in each of the 3 0.75 aliquots. Average the readings and multiply by 3 to determine the concentration in the 2.25 ml lipid extract. Since 75% of the original chloroform-methanol, 2:1 was pipetted in the lipid extraction procedure (15 ml out of 20 ml) multiply by 1.33 to determine the concentration of alpha-tocopherol per g liver tissue. For testes, the final value is divided by the weight of the testes tissue to determine the weight per g.

5. Determine the concentration of beta-carotene by interpolating the actual concentration of beta-carotene in 0.75 ml with the spectrophotometric reading of the sample at 455 mμ. This value gives the concentration of beta-carotene in each of the 3 0.75 ml aliquots. Average the readings and multiply by 3 to determine the concentration of beta-carotene in the 2.25 ml lipid extract. Since 75% of the original chloroform-methanol, 2:1 was pipetted in the lipid extraction procedure (15 ml out of 20 ml) multiply by 1.33 to determine the concentration of beta-carotene per g of liver tissue. For testes, the final value is divided by the weight of the testes tissue to determine the concentration of beta-carotene per g of testes tissue.

6. In order to test the recovery of alpha-tocopherol from whole tissue, 3 known amounts of alpha-tocopherol[10] were added to 3 separate 0.75 ml aliquots of serum (ie. from 3 different samples) and the results were compared with the average of the other 2 0.75 ml aliquots. The known amounts were prepared by taking a 0.75 ml aliquot of alpha-tocopherol in 100% ethanol from the appropriate tube in the set of standard solutions. The 0.75 ml of alpha-tocopherol in ethanol was added to the samples instead of the 0.75 ml of 100% ethanol as outlined in step 3 of the procedure for serum alpha-tocopherol.

No[11]	ml lipid extract	ml d H_2O	ml 100% EtOH	ml 3.75 μg alpha-tocopherol in .75 ml	ml 7.5 μg alpha-tocopherol in .75 ml	μg alpha-tocopherol / gram liver	% Recovery of alpha-tocopherol
+14,18	0.75	0.75	0.75	-	-		
+14,18	0.75	0.75	0.75	-	-	42.0	
+14,18	0.75	0.75	-	0.75	-	45.5	70%
-3, 11	0.75	0.75	0.75	-	-		
-3, 11	0.75	0.75	0.75	-	-	9.1	
-3, 11	0.75	0.75	-	-	0.75	17.6	85%
+2, 12	0.75	0.75	0.75	-	-		
+2, 12	0.75	0.75	0.75	-	-	27.5	
+2, 12	0.75	0.75	-	-	0.75	36.3	90%

The recovery of alpha-tocopherol was lowest (70%) for the smaller amount of alpha-tocopherol added (3.75 μg). For the higher amount of alpha-tocopherol added (7.5 μg) the recovery was good, averaging 87.5%.

FOOTNOTES

1. PR-6 Centrifuge, International Equipment Co.
2. 5°C.
3. The paired water blanks are not read at 455 mμ.
4. Once the ferric chloride reagent has been added, even to a water blank, the red color deepens proportionately over time. This is why a blank must be prepared for each sample.
5. The ratio of beta-carotene at 510 mμ to beta-carotene at 455 mμ was found to be 0.25. This compares with a ratio of 0.29 determined by Quaife et al (131).
6. For greater ease of calculation determine by interpolation the value of D_{510} alpha-tocopherol standard at 1.0 mg%.
7. To prevent oxidation of the lipids, butylated hydroxytoluene (1 g/l) was added to all chloroform:methanol used in this procedure.
8. The ratio of beta-carotene at 510 mμ to beta-carotene at 455 mμ was found to be 0.25. This compares with a ratio of 0.29 determined by Quaife et al (131).

Footnotes Continued

9. Use the D_{510} (spectrophotometric reading) of one of the values from the alpha-tocopherol standard curve and determine by interpolation the concentration in 0.75 ml.

10. 3.75 μg, 7.5 μg and 7.5 μg.

11. Indicates from (+) control or (-) experimental group. All samples were from liver tissue.

APPENDIX VIII

Negative Staining Technique for Electron Microscopy

1. Using a clean Pasteur pipette for each sample to be tested, take one drop of the diluted subcellular membrane pellet suspension (pellet plus distilled water to 4 ml) and place on a clean glass microscope slide.
2. Add 4 drops of distilled water.
3. Add 1 drop of 1% phosphotungstic acid.
4. Using a pair of watchmakers tweezers remove copper electron microscope disc from its parafilm coating, touching only the edges of the disc.
5. Take 1 drop of stained sample and carefully place on copper grid. Wait 5-10 sec, then carefully blot off any excess fluid by placing a filter paper alongside the copper disc and allowing the excess fluid to absorb onto the paper.
6. Carefully place stained copper grid inside a labelled petri dish which has been lined with a piece of filter paper. Cover and store.
7. Take electron microscope pictures within 10 days.

APPENDIX IX

Method for Lipid Extraction

A. Method for Extraction of Phospho[redacted]s from Subcellular Membrane Pellet

1. Pipet 2 1 ml portions of resuspended[1] SM pellet into 2 labelled 25 ml pyrex round-bottomed centrifuge tubes. Pour the third 1 ml portion into a third 25 ml pyrex round-bottomed centrifuge tube.

2. To each tube add 20 ml of chloroform-methanol, 2:1[2] and cap with a pyrex ground glass stopper.

3. Shake vigorously for 2 min. Time exactly using a second hand. Pause once or twice to remove the cap and release any air pressure.

4. To each tube add 4.0 ml 0.75% NaCl[3] in water. Shake vigorously for 20 sec.

5. Centrifuge for 10 min at 1500 r.p.m.'s in a refrigerated centrifuge[4].

6. Using a clean Pasteur pipette for each sample, carefully remove the bottom layer of chloroform-methanol and place into a clean, labelled 25 ml pyrex flask. (In practice, it was found that the last 2-3 ml of chloroform-methanol were difficult to remove without disturbing the upper phase, therefore 15 ml out of the total 20 ml were removed.)

7. Evaporate samples under nitrogen until one thick, oily droplet remains[5].

8. Resuspend droplet in 0.25 ml chloroform-methanol, 2:1 and, using a clean Pasteur pipette, transfer to a clean, labelled 1 dr vial.

9. Seal under nitrogen, taking care not to evaporate (concentrate) the sample or, splash any sample up the sides of the vial. Cap. Cover with tape.

10. Store overnight at -60°C.

B. Method for Extraction of Tissue Lipid for Tissue Alpha-Tocopherol Determination

NB. Because of the light sensitive behavior of vitamin E, all analytical procedures involving alpha-tocopherol standards or samples, including the initial lipid extraction for tissue alpha-tocopherol determination were conducted with the artificial room lighting turned off and the window blinds closed.

1. Place 1 g of liver tissue[6] into a pyrex Potter-Elvehjem homogenizer. Add chloroform-methanol, 2:1[7] to 10 ml and homogenize thoroughly by hand for 3 min. This time period was found to be adequate for complete homogenization.

2. Pour homogenate through a layer of clean gauze into a 25 ml pyrex round-bottomed centrifuge tube. Add an additional 10 ml chloroform-methanol, 2:1 and cap with a pyrex ground glass stopper.

3. Shake vigorously for 2 min, pausing once or twice to remove the cap and release any air pressure.

4. Add 4 ml 0.73% NaCl in water. Replace glass stopper and shake vigorously for 30 sec.

5. Centrifuge for 10 min at 2400 r.p.m.'s in a refrigerated centrifuge[8].

6. Using a clean Pasteur pipette for each sample, carefully remove 15 ml of the bottom phase of chloroform-methanol, 2:1 and place into a clean, labelled 25 ml flask.

7. Reduce the volume of the extract by evaporating the chloroform-methanol under a flow of nitrogen until 1 thick oily droplet remains.

8. Resuspend droplet in 2.25 ml of chloroform-methanol, 2:1 and, using a clean Pasteur pipette, transfer to a clean, 5 dr vial.

9. Seal under nitrogen, taking care not to evaporate (concentrate) the sample or splash any sample up the sides of the vial. Cap. Cover with tape.

10. Store overnight at -60°C.

FOOTNOTES

1. The SM pellet was resuspended up to 4 ml in distilled water and 1 ml was set aside for Lowry protein determination, leaving 3 ml.

2. To prevent oxidation of the phospholipids, butylated hydroxytoluene (1 g/l) was added to all chloroform-methanol, 2:1 used in this procedure.

3. Weigh out 0.73 g NaCl. Place in beaker and add distilled water to 100 ml. Mix thoroughly. In practice it was found convenient to mix up 250 ml portions using 1.83 g.

4. PR-6 Centrifuge maintained at 5°C.

5. Care was taken not to dry the sample completely, as this has been found to drastically decrease PE levels (139).

6. Or 1 testis weighing approximately 1.5 g.

7. Butylated hydroxytoluene (1 g/l) was added to all chloroform-methanol, 2:1 used in this procedure.

8. PR-6 Centrifuge maintained at 5°C.

APPENDIX X

Determination of Subcellular Membrane Pellet Protein Level.

A. Preparation of Reagents:

1. Na_2CO_3/NaOH mixture. Mix fresh each day making up 4 g Na_2CO_3 in 200 ml 0.1N NaOH[1]. Add 2 ml 2% NaK Tartrate[2] and 2 ml 1% $CuSO_4$[3] in that order.

2. 1N Folin reagent. Mix fresh each day by adding distilled water to an equal volume of 2N Phenol. Mix well.

B. Preparation of Standard Solutions:

1. Mix up stock standard solution of Bovine Serum Albumin (25 mg/100 ml) and freeze in 2 ml portions.

2. Make a fresh set of standard solutions for each day of analysis. Prepare triplicate aliquots according to the following table.

Tube #	Concentration µg/0.2 ml	mls BSA 250 µg/ml	mls distilled water
1	0	0.00	0.20
2	10	0.04	0.16
3	20	0.08	0.12
4	30	0.12	0.08
5	40	0.16	0.04
6	50	0.20	0.00

3. Treat as for samples.

C. Procedure

1. For each sample to be analyzed prepare 1:40 dilution by adding 0.1 ml of homogenate[4] to appropriately labelled 100 x 15 mm pyrex test tube containing 3.9 ml distilled water. Mix tube vigorously for 5 sec on vortex mixer.

2. Using a clean pipet for each sample, pipet three 0.2 ml portions of the 1:40 dilution into 3 100 x 15 mm tubes for triplicate analyses.

3. To each tube add 2.0 ml of Na_2CO_3/NaOH mixture. Mix each tube lightly

on a vortex mixer. Set aside for 10 min.

4. To each tube add 0.2 ml of 1N Folin reagent. Mix immediately for 5 sec on a vortex mixer. Set aside for 30 min.

5. After 30 min immediately read the absorption on a double-beam spectrophotometer set at 500 m μ.

6. Zero the instrument with a distilled water blank to which has been added all reagents. Read all tubes against the blank.

D. Calculations

1. Average the triplicate spectrophotometric readings of the tubes containing BSA standard and plot against concentration of BSA (see Figure 12).

2. Determine by interpolation the amount of protein in the sample tubes and report values as an average of three tubes.

$$\frac{\text{mg protein}}{\text{g tissue}} = \frac{\frac{\mu\text{g protein}^5}{.2\text{ ml}} \times 5 \times 40 \times 4}{\text{wt tissue}} \times .001$$

$$= \frac{\frac{\mu\text{g protein}}{.2\text{ ml}} \times 800}{\text{wt tissue}} \times .001$$

FOOTNOTES

1. 8g NaOH in 200 ml distilled water.

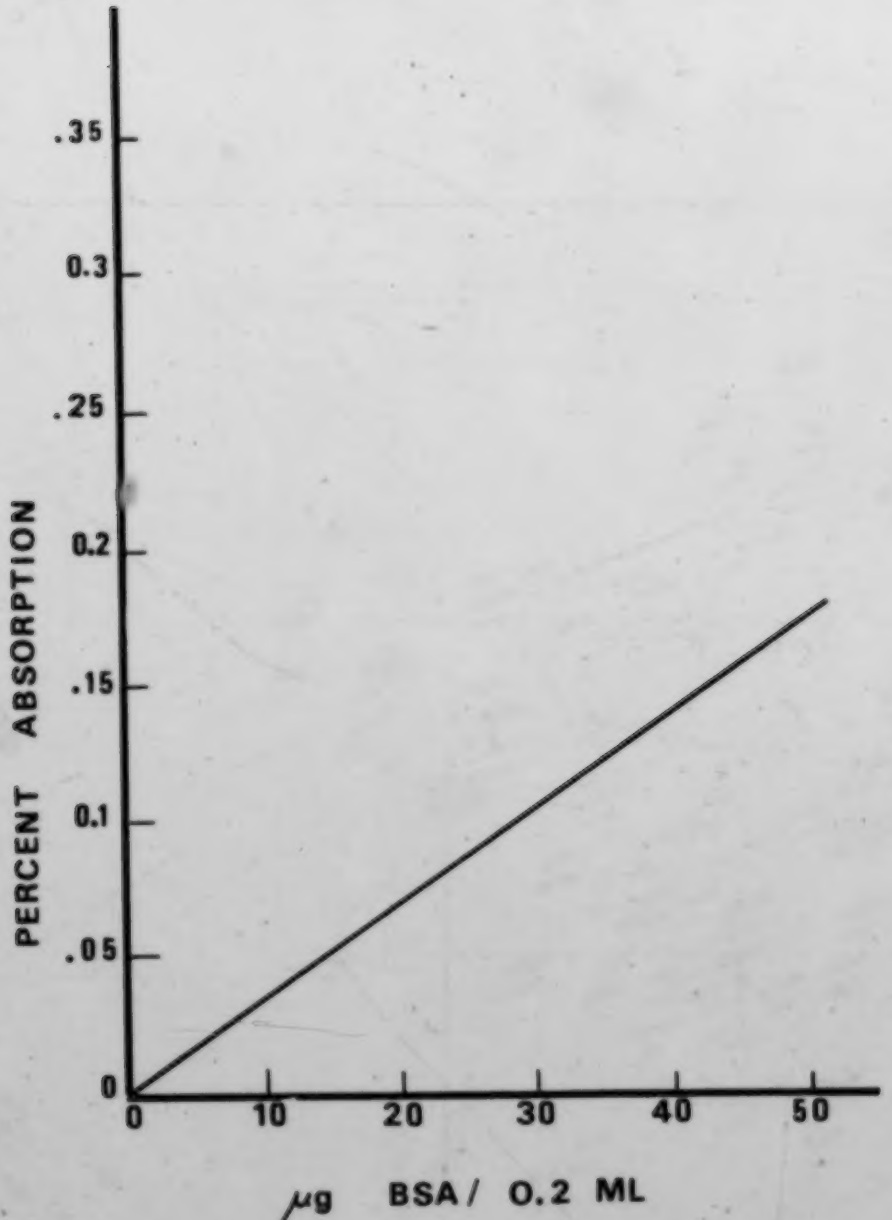
2. 2 g NaK Tartrate in 100 ml distilled water.

3. 1 g $CuSO_4$ in 100 ml distilled water.

4. In 0.1 ml liver homogenate there is present the SM obtained from 25 mg whole liver tissue, while in 0.1 ml testes homogenate is the SM obtained from 50 mg whole testes tissue.

5. Determined by interpolation.

Figure 12. Bovine serum albumin standard curve.



APPENDIX XI

Thin Layer Chromatographic Separation of Phosphatidyl Choline, Phosphatidyl Ethanolamine and Diphosphatidyl Glycerol from Subcellular Membrane Lipid Extract

A. Preparation of Chromatoplates

1. For each chromatoplate[1] lightly score the centre point of each side with a glass cutter[2]. Using the sharp side of a metal ruler, and pressing lightly on the silica gel, make a score line between each opposing centre point. This will form a cross, and will divide the plate into four plates of 10 x 10 cm.

2. Taking great care not to touch any of the silica gel, lay the sharp side of the ruler directly alongside each score line and score with a sharp glass cutter, using the ruler as a tread for the cutter.

3. Place the plate over a heavy book, lining up the scored line just past the edge of the book. Taking care not to disturb the silica gel, give one or more sharp blows to the outer edge of the plate. The plate should fracture into 2 10 x 20 cm plates. Repeat with each plate until four 10 x 10 cm plates have been made from the original plate[3].

B. Preparation of the Solvent Mixture

1. Line 3 chromatographic chambers (29 x 10 x 27.5 cm) with filter paper. About 30 min before the run, pour the following solvent mixtures over the filter paper, and allow about 0.75 to 1.00 cm of solvent to pool at the bottom of each tank.

Tank 1 (for First Direction Solvent System)	
chloroform[4]	130 ml
methanol	60 ml
40% methylamine[5]	15 ml

Add, in order on previous page, to a 250 ml round-bottomed flask with a pyrex stopper. This mixture can be kept for several days. Before each run, check the tank to make sure it contains sufficient solvent. Each week, clean tank thoroughly and prepare fresh solvent mixture.

Tank 2 (for the Intermediary[6] Solvent System)	
diethylether	190 ml
glacial acetic acid	10 ml

Add, in above order, to a 250 ml round-bottomed flask with a pyrex stopper. Prepare fresh each day and clean tank after each day of use.

Tank 3 (for the Second Direction Solvent System)	
chloroform	100 ml
acetone	40 ml
methanol	20 ml
acetic acid	30 ml
water	10 ml

Add, in above order, to a 250 ml round-bottomed flask with a pyrex stopper. Swirl flask after each addition to prevent phase partitioning. This mixture can be kept for several days. Before each run, check the tank to make sure it contains sufficient solvent. Each week, clean tank thoroughly and prepare fresh solvent mixture.

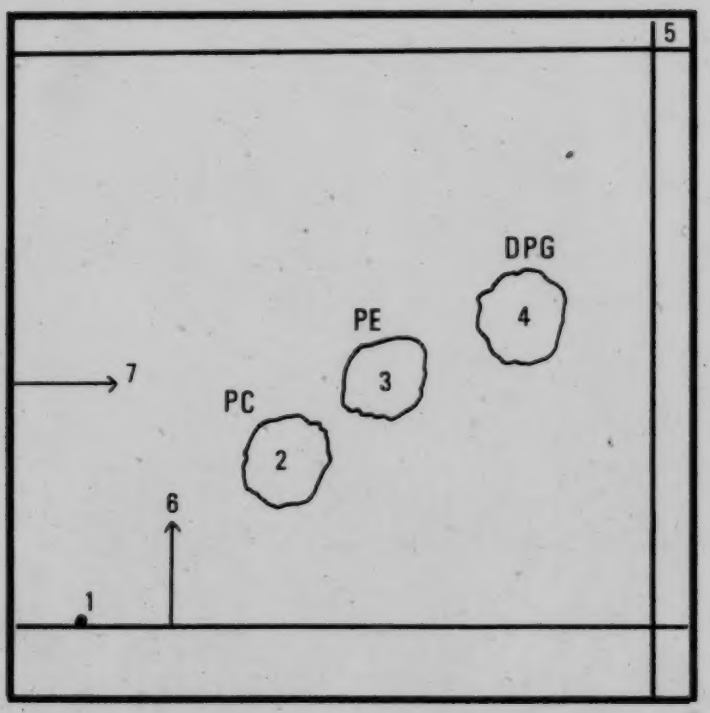
C. Spotting Procedure

1. Line up the 10 x 10 cm chromatoplates so that the 2 straight edges lie across the bottom and along the left side. This ensures that an irregular edge will not be placed into the solvent, causing an irregular solvent front and disorderly migration of lipids.

2. Lightly mark the point of application at the left hand corner (see Figure 13), 1 cm from the bottom of the plate and 1 cm from the left of the plate.

3. Label the plate in the upper right corner. This area of the plate lies past the solvent front for both directions.

Figure 13. Thin-layer chromatographic separation of rat liver and testes subcellular membrane lipid extract.



1. Origin
2. PC Rf = 2.4[7]
3. PE Rf = 3.6[8]
4. DPG Rf = 4.5[9]
5. Plate label
6. First Direction
7. Second Direction

4. Place the one dram vials containing samples into a bucket filled with ice, and, using a micropipette, carefully spot 20 μl of lipid extract from each sample (3 lipid extracts for each sample) onto the appropriate plate[10]. For each day of chromatography, run 3 plates[11] of each standard to test for recovery. As a precaution in case any samples need to be rerun, reseal all samples under nitrogen and cap securely before storing at -60°C.

D. Development of the Plates

1. Make certain that the sample (or standard) has been allowed to dry thoroughly[12] before developing the plate.

2. Using long forceps, carefully place the plates, 4 at a time, into the first solvent system. Develop for about 15 min to the point just below the marking line. Remove the plates and air dry.

3. Expose the plates to the fumes of a concentrated HCl solution for 3 min[13]. Remove the plates. Dry for 3 min with the plates positioned in a fume hood, and a further 2 min with the plates exposed to ambient room air.

4. Place the plates, 4 at a time, into the second solvent system with the origin now at the lower right corner. Develop for about 10 min until the solvent front just reaches the marking line. Remove from the chamber and air dry for 3 min.

5. Rerun the plates in the same direction in the third solvent system. Develop for about 15 min until the solvent front reaches the same line as for the second solvent system. Remove the plates from the chamber and dry thoroughly.

6. Place the plates into a chamber filled with iodine crystals[14]. Leave long enough to be able to visualize outline of phospholipid spots.

FOOTNOTES

1. Precoated silica gel G plates 250μ thick, and 20 x 20 cm were purchased from Analtech Co.

2. Throughout this procedure, and during the development of the plates when they are drying outside the solvent chambers, all plates are placed on a clean sheet of paper to prevent contamination.

3. About half the plates cleaved with good, straight fracture lines. Half of the remaining plates were kept because the fracture line, while crooked, did not break into the plate more than 0.5 cm. The remaining quarter were discarded.

4. Butylated hydroxytoluene (1 g/l) was added to all chloroform used in the chromatography.

5. Weigh out 40 g methylamine. Place in flask and add distilled water to 100 ml. Mix thoroughly.

6. Plates in the Intermediary tank also are run in the second direction. The Intermediary run, however, is not strictly necessary, but was used throughout the chromatography procedure.

7. This Rf compares with that of Yavin and Zutra (176).

8. This Rf was close (3.8) to that of Yavin and Zutra (176).

9. The Rf for DPG was higher (4.5 vs 3.5) than that of Yavin and Zutra (176).

10. The plates can be used without prior heat activation.

11. Spot the same quantity 3 times.

12. The plates were dried at room temperature, 20°C.

13. This was accomplished by setting four small petri dishes upside down on the bottom of a fourth chromatography chamber and pouring conc. HCl into the tank so it just covered the bottom. The plates were placed onto the petri dishes and left for 3 min.

14. This chamber must be well sealed and kept in the fume hood to prevent any escape of the toxic iodine vapors.

APPENDIX XII

Phosphorus Method for Quantification of Phospholipids

A. Preparation of Reagents

1. 60% perchloric acid - slowly pour 85 ml perchloric acid into 15 ml water.

2. 5% ammonium molybdate in 4 N H_2SO_4 - weigh out 5 g of ammonium molybdate. Place in flask. Add 4 N H_2SO_4[1] to 100 ml. Swirl to mix.

3. extraction solution - add 20 parts petroleum ether to 80 parts isobutanol.

4. $SnCl_2$ - sulphuric acid reagent. Mix up a stock solution of 43 g stannous chloride in conc. HCl. For each day of analysis prepare reagent by adding 10 μl stock solution to 4.0 ml conc. H_2SO_4.

B. Preparation of Standards

1. For each chromatographic run of samples, spot in triplicate a known amount of phospholipid for plates to be run as standards. Spot the same amount of standard 3 times[2]. Develop plates exactly as for the samples. Quantitate as for samples (see part C and D of this Appendix).

2. Prepare a phosphorus standard curve. Pipet triplicate aliquots of PC[3] in the following amounts: 10 μl, 20 μl, 40 μl, 60 μl and 80 μl. This corresponds to concentrations of 10 μg, 20 μg, 40 μg, 60 μg and 80 μg. Pipet 3 25 ml aliquots of chloroform directly into 10 ml pyrex test tubes[4] for the 0 μg concentration.

3. Dry all tubes under a nitrogen source to remove the chloroform solvent.

C. Procedure

1. Carefully scrape off the silica gel G absorbant containing the phospholipid spot from all sample and standard chromatographic plates. Remove the same surface area of absorbant[5] for each phospholipid.

2. Place each phospholipid spot into a separate 10 ml pyrex test tube.

3. To all tubes[6] add 0.2 ml of 60% purchloric acid.

4. Place tubes into a sand bath and heat at 180°C for approximately 30 min until all tubes are completely digested. The contents of the tubes initially will turn black, then brown, then amber and then pale yellow. Digestion is completed when the color is as clear as distilled water.

5. Cool to room temperature.

6. Add 2 ml distilled water to each tube. Mix on a vortex mixer for 5 sec.

7. Add 0.5 ml of molybdate solution. Mix on a vortex mixer for 5 sec.

8. Add 2.5 ml extraction solution. Mix on a vortex mixer for 15 sec.

9. Centrifuge[7] under refrigeration for 10 min at 1500 r.p.m.'s.

10. Pipet 2 ml top layer from each tube into correspondingly labelled clean 10 ml pyrex test tubes.

11. To each 2 ml aliquot of top layer add 0.3 ml absolute ethanol. Mix on a vortex mixer for 5 sec.

12. Add 0.2 ml stannous-chloride reagent. Mix on a vortex mixer for 5 sec.

13. Set tubes aside for 15 min. A blue color will develop.

14. Read the absorption of each sample on a double-beam spectrophotometer set at 625 mμ. Zero the instrument with a blank to which has been added all reagents[8]. Read all tubes against the blank.

D. Calculations

1. Subtract the absorbance of the blank from the absorbance of all standard and sample readings.

2. Average the triplicate spectrophotometric readings of the tubes containing PC standard and plot against the concentration of PC in μg/μl (see Figure 14).

3. For purposes of simplicity, the molecular weight of PC and PE were assumed to be 800[9]. The molecular weight of DPG was assumed to be 1600[10]. The quantity of standard in μg is directly proportioned to the amount of phosphorus, therefore to determine the quantity of PC or PE in a sample, it is possible to interpolate directly from the standard curve. The value must be halved for DPG which has 2 moles of phosphorus for every mole of phosphorus in PC or PE.

4. Average the 3 readings. Compare the recovery of spotted phospholipid standards with the values for non-spotted standards ie. the standard curve.

5. Determine the value of μg SM phospholipid per g whole tissue in the samples according to the following formula (see Figure 15):

$$n^{11} \times 12.5^{12} \times 3^{13} \times 1.33^{14} \times 1.33^{15} = X$$

$$\therefore \quad n \times 66^{16} = X$$

Adjust the value according to the calculated recovery of phospholipid standard.

For testes tissue divide by the weight of the testes to obtain the quantity of SM phospholipid in μg per g whole testes.

E. Recovery of Phospholipids

1. On each day of chromatography, standards were spotted, developed and quantitated, and the results were compared with the values of the non-spotted standards as previously outlined. This was undertaken to test

Figure 14. Phosphorus standard curve.

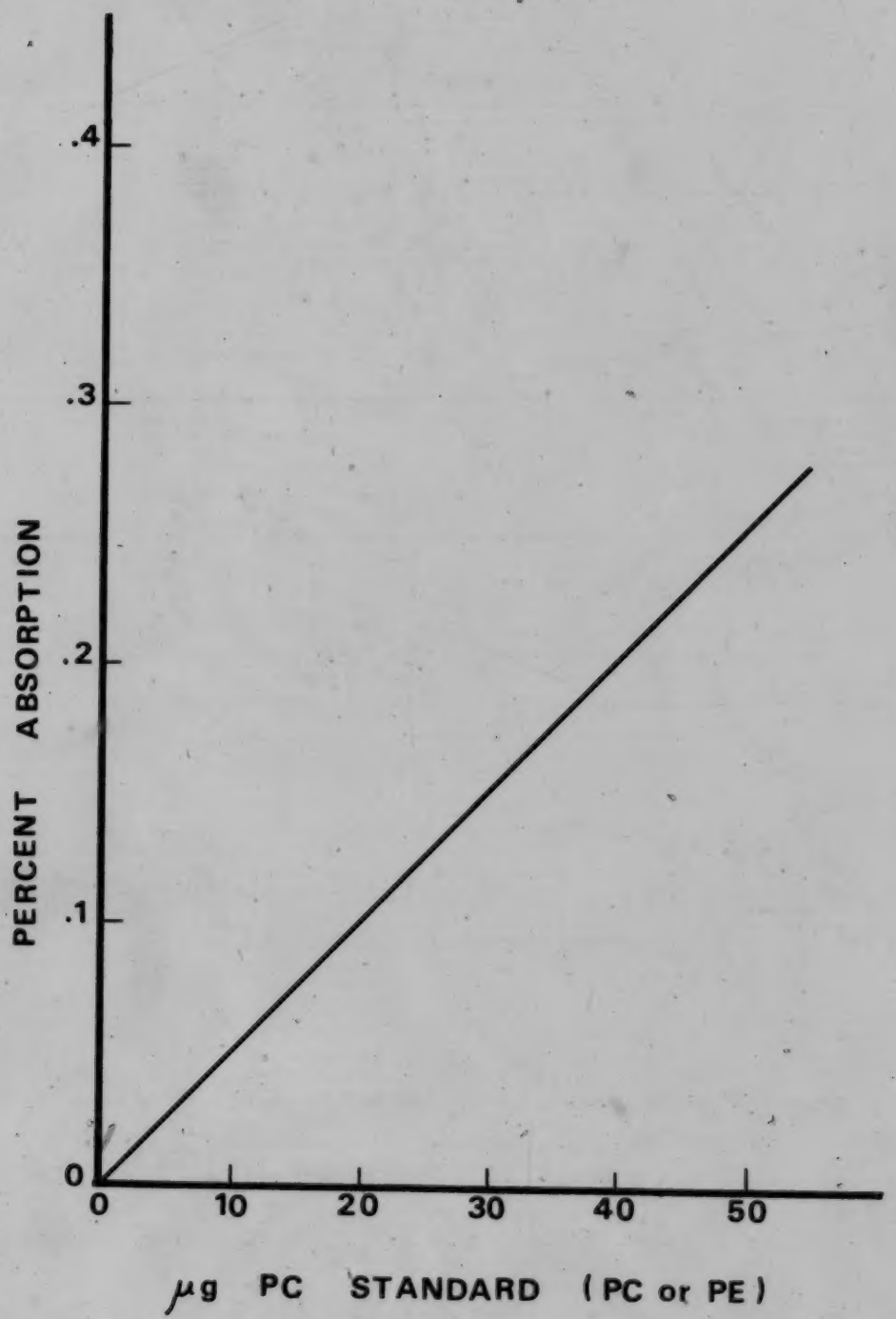
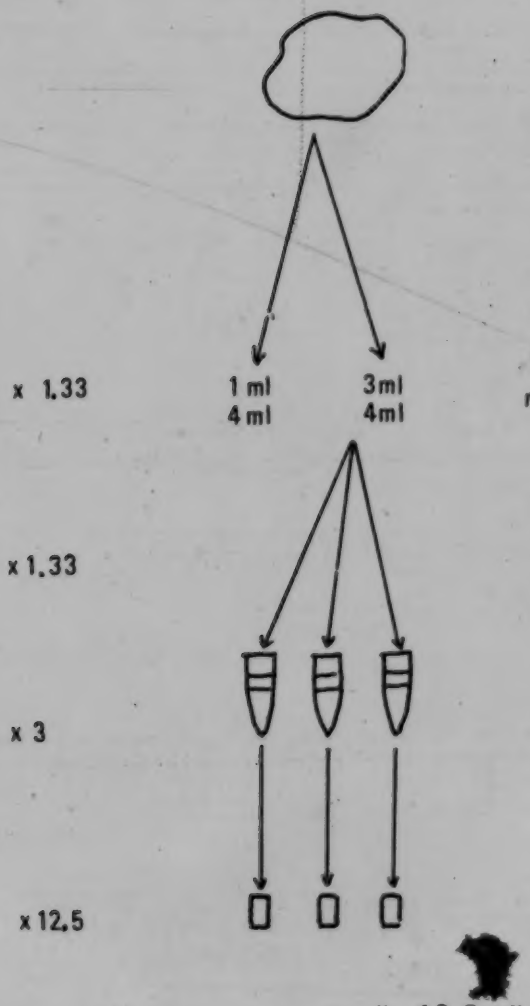


Figure 15. Determination of subcellular membrane phospholipid per gram whole tissue.


1 g liver
3ml/4ml SM extract used for 3 x 1 ml lipid extraction (each) in 20 ml chloroform–methanol, 2:1
x 1.33
1 ml
4 ml
3ml
4ml
x 1.33
15ml/20ml pipetted out and each reduced to 250 µl
x 3
750 µl total extract
20µl / 250µl spotted on chromatographic plate
x 12.5

$$x = n \times 12.5 \times 3 \times 1.33 \times 1.33$$

$$\therefore x = n \times 66$$

the day-to-day variations in phospholipid recovery from the chromatography procedure itself.

Recovery averaged 73%, but did drop to as low as 66% on one day of analysis, and was as good as 80%.

2. Recovery also was tested by adding a known quantity of phospholipid standard to 3 different samples prior to the perchloric acid digestion step. Recovery was calculated by comparing the average value of the 2 other samples with the value of the sample plus standard. Recovery averaged 86% and is outlined in the table below.

Sample No	g PC standard added	Interpolated reading for PC-average of 2 samples	Interpolated reading of sample plus standard	% recovery of added phospholipid
-L 8, 13[17]	-	38		
-L 8, 13	-			
-L 8, 13	20		51	79%
+L 4, 5[18]	-	40		
+L 4, 5	-			
+L 4, 5	20		54	90%
+T 16, 17[19]	-	28		
+T 16, 17	-			
+T 16, 17	20		42	89%

3. An important component to evaluating the recovery of phospholipids was to compare the phospholipid levels from the liver and testes SM of previously frozen tissue with the phospholipid levels from the liver and testes SM of a freshly sacrificed animal[20]. The recovery was tested on the same day that samples 4, 5 and 14, 18 were chromatographed, thus the values were compared with those particular samples. When compared with the above 2 control samples the levels of phospholipid for the liver SM of the freshly killed rat were 1.10 times higher for PC, 1.27 times higher for PE and 1.33 times higher for DPG. This indicates that, relative to the freshly killed rat, the recovery of liver SM phospholipids from control samples 4, 5 and 14, 18 (averaged) representing the pre-

viously frozen tissue was 91% for PC, 79% for PE and 75% for DPG. When compared with control samples 4, 5 and 14, 18, the levels of phospholipid for the testes SM of the freshly killed rat were 1.16 times higher for PC, 1.20 times higher for PE and 1.42 times higher for DPG. This indicates that relative to the freshly killed rat, the recovery of testes SM phospholipids from the 2 control samples (averaged), representing previously frozen tissue, was 86% for PC, 83% for PE and 70% for DPG (see table below).

Tissue	Phospholipid	Average for control samples[21] (from frozen tissue)	Average for samples from freshly killed rat	% recovery of frozen tissue
Liver SM	PC	4.418	4.860	91%
	PE	2.270	2.882	79%
	DPG	1.714	2.280	75%
Testes SM	PC	3.716	4.301	86%
	PE	2.511	3.013	83%
	DPG	1.391	1.975	70%

When the values for liver SM phospholipids of the freshly killed rat were compared with the averaged results of the liver SM phospholipid from all the control rats, the values were 1.27 times higher for PC, 1.56 times higher for PE and 2.22 times higher for DPG. Thus, the recovery of liver SM phospholipids from all the control samples (averaged) was 79% for PC, 64% for PE and 45% for DPG, relative to the levels in the freshly killed rat. Similarily, when the values for testes SM phospholipids of the freshly killed rat were compared with the averaged results of the testes SM phospholipids from all the control rats, the values were 1.72 times higher for PC, 2.04 times higher for PE and 2.78 times higher for DPG. Thus, the recovery of testes SM phospholipids from all the control samples (averaged) was 58% for PC, 49% for PE and 36% for DPG, relative to the levels in the freshly killed rat (see table on the following page).

Tissue	Phospholipid	Average for control samples (from frozen tissue)	Average for samples from freshly killed rat	% recovery of frozen tissue
Liver SM	PC	3.830	4.860	79%
	PE	1.838	2.882	64%
	DPG	1.031	2.280	45%
Testis SM	PC	2.475	4.301	58%
	PE	1.490	3.013	49%
	DPG	0.715	1.975	36%

FOOTNOTES

1. Prepare by adding 225 ml conc. H_2SO_4 to 100 ml distilled water.
2. In practice, 2 standards were always run since it was quite time consuming to always spot a set of each standard. PC always was used as 1 standard, and a second standard (alternating between PE and DPG) also was run.
3. From a stock solution of 1 μg/ μl PC in chloroform - butylated hydroxytoluene (1 g/l) was added to all chloroform used in this procedure.
4. All tubes used in this procedure wer acid washed in 1N HCl to remove any traces of phosphate soap that would alter the results.
5. 4.0 cm^2, usually a 2.0 cm x 2.0 cm square.
6. Includes all tubes containing phospholipids from silica gel G scrapings (spotted standards and samples) and standards containing dried PC.
7. PR-6 Centrifuge, International Equipment Co. maintained at 5 C.
8. 2.0 ml extraction solution, 0.3 ml absolute ethanol, 0.2 ml stannous chloride reagent.
9. The actual values vary from 750 to 850 depending on the type of fatty acids present.
10. The molecular weight of standard was 1626, but since the molecular weight of the DPG of the rat SM could vary from 1550 to 1650, depending on the type of fatty acids, it was assumed to be exactly double the weight of the other 2 phospholipids.
11. Where n = average of 3 values interpolated from standard curve.
12. Represents 20 μl/250 μl sample spotted.
13. Represents 3 x 250 μl for total amount of extract.

Footnotes Continued

14. Represents 15 ml/20 ml pipetted from lipid extract.

15. Represents 3 ml/4 ml original homogenate used in lipid extracts.

16. 20 μl spot represents 0.0152 liver tissue. 66 x 0.0152 g = 1.0 g.

17. Liver tissue, pair #8, 13, experimental group.

18. Liver tissue, pair #4, 5, control group.

19. Testis tissue, pair #16, 17, control group.

20. Preparation of the liver and testes SM pellet was undertaken as previously described.

21. Rats 4, 5 and 14, 18.

APPENDIX XIII

Table of Food Consumption of Control and Tocopherol-Deficient Rats Throughout Feeding Trial

	Food Consumption[1]									
	Number 1		Number 2		Number 3		Number 4		Number 5	
Week	C[2]	E[3]	C	E	C	E	C	E	C	E
0	12	12	12	12	13	13	12	12	13	13
1	14	14	14	14	15	15	15	15	14	14
2	15	17	15	16	17	17	17	17	17	17
3	17	17	20	21	20	20	19	19	18	18
4	19	18	18	19	22	19	20	18	22	18
5	19	19	19	18	21	19	19	17	21	17
6	18	19	19	18	20	18	19	18	19	18
7	19	19	19	18	21	19	20	18	19	18
8	19	20	19	19	20	19	19	19	20	21
9	21	22	19	21	20	21	20	20	21	22
10	22	24	21	24	22	24	23	23	22	24
11	22	23	21	21	23	22	24	24	25	24
12	24	24	23	25	24	24	22	24	24	24
13	24	24	23	23	22	25	23	23	23	25
14	24	24	25	24	24	24	25	23	25	24
15	25	24	25	24	25	25	27	24	25	25
16	23	24	23	23	25	24	26	24	24	25
17	23	24	23	24	25	24	26	24	25	23
18	24	21	23	24	24	23	26	24	25	24
19	24	24	24	25	24	23	23	19	25	24
20	24	25	24	24	20	22	25	25	25	25
21	25	26	26	25	24	24	25	27	25	23
22	26	26	26	27	26	24	25	25	28	27
23	26	21	26	27	26	26	25	24	26	27
Final Week	26	26	25	26	26	26	24	23	27	27

Food Consumption[1]

Week	Number 6		Number 7		Number 8		Number 9		Number 10	
	C[2]	E[3]	C	E	C	E	C	E	C	E
0	12	12	12	12	13	13	12	12	12	12
1	15	15	15	15	15	15	14	14	15	15
2	16	16	16	17	17	17	16	17	16	16
3	18	19	19	20	19	19	19	20	19	19
4	20	18	21	19	22	21	21	22	20	20
5	18	19	21	18	17	20	20	20	20	17
6	22	18	20	19	21	17	20	21	21	17
7	22	18	21	19	20	18	21	20	21	18
8	19	19	20	20	21	18	20	20	19	19
9	20	21	21	21	21	21	22	22	20	21
10	22	24	23	24	23	23	23	22	23	23
11	23	23	23	22	23	24	23	24	22	23
12	24	24	24	25	26	24	26	26	21	24
13	24	24	24	24	24	24	21	25	23	25
14	21	23	24	24	24	24	26	24	26	24
15	21	23	24	24	25	23	23	24	23	25
16	23	24	24	24	25	24	24	24	24	25
17	24	23	24	24	25	23	24	24	25	24
18	24	25	23	24	24	25	25	24	25	21
19	21	22	23	23	24	22	25	22	21	21
20	25	25	23	25	24	24	25	25	23	26
21	25	27	24	26	24	23	27	22	25	27
22	26	26	25	25	26	24	27	24	25	27
23	27	26	26	26	26	27	26	26	25	24
Final Week	25	25	26	26	27	23	26	26	25	26

Food Consumption[1]

Week	Number 11		Number 12		Number 13		Number 14		Number 15	
	C[2]	E[3]	C	E	C	E	C	E	C	E
0	12	12	13	13	12	12	12	13	13	13
1	14	14	15	15	15	15	16	16	15	15
2	16	17	17	17	17	17	17	17	17	17
3	19	19	20	20	19	19	18	18	19	19
4	21	21	22	20	20	20	21	21	22	21
5	20	21	21	20	19	17	20	20	22	20
6	19	19	20	21	20	18	20	17	20	18
7	22	19	20	20	19	19	19	18	20	18
8	21	20	20	20	19	10	20	20	20	20
9	21	21	21	22	20	21	20	22	19	22
10	22	23	23	23	24	23	21	23	20	24
11	23	24	24	23	24	23	26	23	22	24
12	26	24	23	24	24	25	25	24	21	25
13	24	25	24	24	23	25	22	23	25	24
14	24	24	24	24	23	24	22	23	24	24
15	24	24	25	23	23	26	24	23	25	24
16	25	24	25	23	23	25	24	23	25	23
17	25	25	25	24	24	24	23	23	25	23
18	25	24	25	24	24	24	24	24	25	21
19	25	20	24	24	25	25	24	20	25	25
20	25	25	23	24	25	27	24	24	25	25
21	26	25	24	23	25	22	25	27	25	26
22	26	25	25	25	26	26	26	26	25	26
23	26	27	25	27	28	27	27	27	27	27
Final Week	25	27	25	26	27	25	25	26	26	27

Food Consumption[1]

Week	Number 16 C[2]	Number 16 E[3]	Number 17 C	Number 17 E	Number 18 C	Number 18 E	Number 19 C	Number 19 E	Number 20 C	Number 20 E	MEAN C	MEAN E
0	13	13	12	13	14	14	13	13	13	14	-	12.7
1	15	15	14	14	16	16	15	15	15	15	-	14.8
2	16	16	17	17	17	17	17	17	17	17	-	16.8
3	19	19	20	20	19	19	19	20	17	17	-	19.1
4	21	20	21	20	22	22	21	20	22	21	20.9	19.9
5	21	20	20	20	21	19	21	20	20	19	20.0	19.0
6	19	19	19	19	21	19	23	19	20	20	20.0	18.6
7	19	19	20	20	21	20	21	19	20	20	20.2	18.9
8	19	19	20	21	21	20	22	20	20	21	19.9	19.8
9	19	21	20	22	21	22	22	21	20	23	20.4	21.5
10	21	23	22	23	22	23	23	23	23	24	22.3	23.4
11	23	24	22	22	22	23	23	24	25	24	23.2	23.2
12	25	26	22	24	24	25	24	25	25	25	23.9	24.6
13	22	25	27	25	26	24	27	24	25	24	23.8	24.3
14	23	25	24	25	24	24	24	24	27	23	24.2	23.9
15	24	24	24	25	25	24	25	24	27	25	24.5	24.2
16	25	24	24	24	24	24	25	24	26	24	24.4	24.0
17	25	23	24	25	25	25	25	24	26	23	24.6	23.8
18	25	20	27	26	25	24	26	25	26	24	24.8	23.6
19	25	24	24	25	25	24	26	25	26	26	24.2	23.2
20	25	24	26	25	25	23	25	26	26	26	24.4	24.8
21	25	25	26	25	25	26	25	26	26	27	25.1	25.1
22	26	26	26	26	25	26	25	26	26	26	25.8	25.7
23	26	22	28	27	26	27	26	26	26	27		25.9
Final Week	27	24	27	27	24	26	26	25	27	27	25.8	25.7

FOOTNOTES

1. Food consumption is expressed to nearest g.
2. Indicates control group.
3. Indicates experimental group

APPENDIX XIV

Table of Weight Gain of Control and Vitamin E-Deficient Rats Throughout Feeding Trial

Week	Weight Gain[1]							
	Number 1		Number 2		Number 3		Number 4	
	C[2]	E[3]	C	E	C	E	C	E
0	63.0	65.0	67.0	67.0	71.0	70.0	72.0	70.0
1	99.0	95.0	97.0	98.0	103.0	101.0	101.0	100.0
2	131.0	134.0	124.0	131.0	135.0	133.0	135.0	134.0
3	167.0	186.5	179.0	198.0	176.0	187.5	162.5	178.0
4	212.5	235.5	231.0	251.0	231.0	237.0	213.5	224.0
5	245.5	277.0	276.0	286.5	278.0	276.0	253.5	258.5
6	268.5	309.0	303.5	318.5	305.5	306.0	281.0	285.5
7	298.0	328.5	326.0	342.5	337.5	327.0	303.5	309.0
8	318.5	350.0	356.0	363.5	364.5	352.5	325.0	326.0
9	348.0	381.0	385.0	391.0	394.0	383.0	344.0	351.0
10	363.0	397.0	402.0	417.0	410.0	401.0	370.0	373.0
11	380.0	383.0	425.0	417.0	430.0	401.0	384.0	387.0
12	380.0	409.0	428.0	438.0	430.0	414.0	391.0	384.0
13	405.0	429.0	449.0	459.0	452.0	430.0	406.0	403.0
14	405.0	426.0	452.0	463.0	458.0	440.0	414.0	404.0
15	400.0	430.0	465.0	461.0	465.0	451.0	421.0	412.0
16	417.0	447.0	475.0	467.0	474.0	457.0	435.0	415.0
17	416.0	444.0	472.0	451.0	467.0	455.0	429.0	404.0
18	401.0	413.0	454.0	417.0	463.0	456.0	408.0	380.0
19	415.0	404.0	464.0	419.0	477.0	471.0	424.0	373.0
20	398.0	388.0	429.0	406.0	462.0	451.0	395.0	340.0
21	404.0	425.0	409.0	425.0	445.0	453.0	413.0	366.0
22	438.0	448.0	446.0	456.0	476.0	478.0	437.0	400.0
23	452.0	472.0	438.0	493.0	471.0	489.0	442.0	426.0
Final Week	469.0	472.0	484.0	501.0	408.0	509.0	464.0	426.0

Weight Gain[1]

Week	Number 5 C[2]	Number 5 E[3]	Number 6 C	Number 6 E	Number 7 C	Number 7 E	Number 8 C	Number 8 E
0	73.0	73.0	75.0	73.0	78.0	75.0	78.0	77.0
1	98.0	98.0	105.0	106.0	105.0	103.0	108.0	106.0
2	132.0	137.0	135.0	134.0	138.0	134.0	136.0	139.0
3	177.0	176.0	185.5	190.0	188.5	195.0	176.0	185.0
4	218.0	218.0	229.0	238.5	234.5	246.0	222.0	227.0
5	271.5	259.5	271.0	278.0	277.5	293.5	271.0	273.0
6	303.5	290.5	287.0	308.0	312.5	329.5	295.5	304.5
7	338.5	316.5	330.0	336.0	349.0	353.0	346.0	322.5
8	362.5	337.5	349.0	352.0	376.0	378.0	376.0	339.6
9	396.0	366.0	371.0	382.0	407.0	393.0	403.0	366.0
10	414.0	386.0	386.0	400.0	426.0	422.0	427.0	386.0
11	435.0	393.0	405.0	410.0	443.0	417.0	437.0	397.0
12	444.0	404.0	402.0	413.0	443.0	437.0	443.0	398.0
13	466.0	416.0	419.0	430.0	466.0	446.0	471.0	411.0
14	478.0	420.0	431.0	440.0	483.0	456.0	483.0	416.0
15	486.0	432.0	434.0	442.0	495.0	459.0	497.0	416.0
16	488.0	438.0	432.0	453.0	498.0	470.0	504.0	423.0
17	477.0	437.0	426.0	452.0	492.0	460.0	503.0	415.0
18	467.0	420.0	413.0	439.0	483.0	446.0	481.0	411.0
19	484.0	412.0	424.0	436.0	485.0	432.0	489.0	413.0
20	450.0	380.0	376.0	404.0	449.0	400.0	453.0	381.0
21	447.0	402.0	392.0	415.0	450.0	421.0	457.0	404.0
22	468.0	429.0	417.0	452.0	476.0	453.0	489.0	435.0
23	491.0	459.0	445.0	485.0	471.0	479.0	492.0	454.0
Final Week	530.0	473.0	470.0	486.0	516.0	483.0	534.0	454.0

Weight Gain[1]

Week	Number 9 C[2]	Number 9 E[3]	Number 10 C	Number 10 E	Number 11 C	Number 11 E	Number 12 C	Number 12 E
0	79.0	79.0	80.0	83.0	81.0	84.0	83.0	84.0
1	107.0	111.0	108.0	110.0	106.0	108.0	108.0	108.0
2	137.0	137.0	136.0	139.0	138.0	141.0	142.0	141.0
3	189.5	203.0	181.0	194.0	182.5	205.0	193.0	202.0
4	247.0	259.0	234.0	246.0	230.0	259.0	246.0	254.0
5	285.5	303.0	278.0	282.0	273.0	309.0	284.0	289.0
6	312.5	340.0	294.0	310.0	290.0	352.0	310.0	332.0
7	332.0	362.5	330.0	337.5	318.0	379.0	332.0	352.0
8	357.0	385.0	346.0	355.0	346.0	402.0	357.0	375.0
9	392.0	409.0	370.0	380.0	378.0	436.0	388.0	408.0
10	418.0	435.0	393.0	412.0	399.0	470.0	412.0	434.0
11	433.0	414.0	405.0	410.0	405.0	477.0	422.0	423.0
12	446.0	443.0	409.0	421.0	418.0	488.0	422.0	428.0
13	469.0	463.0	421.0	432.0	437.0	503.0	434.0	445.0
14	447.0	474.0	437.0	437.0	451.0	518.0	442.0	450.0
15	493.0	474.0	448.0	443.0	456.0	527.0	453.0	446.0
16	491.0	487.0	441.0	455.0	461.0	533.0	459.0	446.0
17	486.0	484.0	434.0	443.0	457.0	529.0	447.0	449.0
18	469.0	456.0	429.0	410.0	445.0	514.0	442.0	428.0
19	483.0	443.0	436.0	394.0	457.0	511.0	450.0	428.0
20	446.0	413.0	414.0	357.0	433.0	468.0	442.0	386.0
21	441.0	440.0	416.0	382.0	447.0	479.0	440.0	422.0
22	475.0	462.0	452.0	424.0	476.0	509.0	476.0	443.0
23	476.0	499.0	451.0	471.0	473.0	550.0	473.0	479.0
Final Week	521.0	505.0	483.0	472.0	506.0	562.0	508.0	483.0

Weight Gain[1]

Week	Number 13 C[2]	Number 13 E[3]	Number 14 C	Number 14 E	Number 15 C	Number 15 E	Number 16 C	Number 16 E
0	83.0	85.0	84.0	86.0	85.0	88.0	88.0	88.0
1	115.0	117.0	113.0	121.0	125.0	119.0	126.0	124.0
2	141.0	142.0	141.0	142.0	140.0	147.0	146.0	142.0
3	182.0	196.5	183.0	187.5	179.0	195.0	193.0	195.0
4	237.5	248.0	237.0	238.0	223.0	243.0	243.0	243.0
5	274.0	293.0	280.0	285.0	270.5	287.5	287.5	289.0
6	301.0	323.0	310.0	319.0	297.5	320.5	322.0	327.0
7	320.0	345.0	333.5	345.5	324.0	343.0	348.0	350.0
8	346.0	373.0	363.5	375.0	346.0	367.0	369.0	374.0
9	373.0	397.0	399.0	410.0	386.0	395.0	410.0	409.0
10	397.0	429.0	406.0	432.0	403.0	418.0	432.0	434.0
11	409.0	428.0	416.0	437.0	401.0	426.0	454.0	427.0
12	414.0	440.0	432.0	436.0	422.0	436.0	459.0	441.0
13	437.0	455.0	452.0	455.0	437.0	450.0	481.0	446.0
14	459.0	457.0	467.0	463.0	462.0	454.0	498.0	454.0
15	467.0	475.0	472.0	462.0	471.0	462.0	504.0	459.0
16	471.0	485.0	480.0	465.0	475.0	466.0	512.0	462.0
17	466.0	469.0	473.0	467.0	470.0	460.0	512.0	443.0
18	456.0	443.0	445.0	457.0	465.0	415.0	497.0	416.0
19	471.0	427.0	466.0	432.0	481.0	447.0	522.0	385.0
20	435.0	402.0	441.0	386.0	462.0	422.0	498.0	366.0
21	439.0	430.0	455.0	422.0	445.0	437.0	505.0	393.0
22	451.0	452.0	478.0	456.0	463.0	459.0	524.0	432.0
23	449.0	497.0	465.0	495.0	445.0	479.0	535.0	476.0
Final Week	503.0	504.0	518.0	504.0	491.0	487.0	569.0	474.0

Weight Gain[1]

Week	Number 17 C[2]	Number 17 E[3]	Number 18 C	Number 18 E	Number 19 C	Number 19 E	Number 20 C	Number 20 E	MEAN C	MEAN E
0	90.0	96.0	91.0	97.0	96.0	98.0	98.0	105.0	80.8	82.2
1	119.0	127.0	125.0	123.0	122.0	116.0	123.0	129.0	110.7	111.0
2	145.0	149.0	151.0	149.0	148.0	144.0	151.0	147.0	139.1	139.6
3	201.0	207.0	196.0	212.5	198.0	203.0	198.0	205.0	184.4	195.1
4	257.0	268.0	254.0	269.0	244.0	246.0	245.0	252.0	234.5	245.1
5	302.0	310.0	303.0	305.0	282.0	284.0	298.0	293.0	278.1	286.6
6	372.0	346.0	325.0	339.0	310.0	312.5	323.0	330.0	304.0	320.1
7	352.0	368.0	353.0	361.0	360.0	337.5	349.0	345.5	334.0	343.1
8	380.0	393.0	375.0	375.0	377.5	362.5	381.0	370.0	358.6	365.3
9	406.0	430.0	404.0	414.0	418.0	371.0	411.0	403.0	389.0	394.0
10	434.0	462.0	429.0	440.0	440.0	406.0	433.0	427.0	410.0	419.0
11	448.0	446.0	435.0	449.0	444.0	419.0	442.0	423.0	423.0	419.0
12	455.0	460.0	434.0	459.0	458.0	428.0	463.0	435.0	430.0	431.0
13	468.0	478.0	449.0	476.0	472.0	449.0	468.0	453.0	448.0	446.0
14	485.0	488.0	465.0	481.0	498.0	453.0	487.0	459.0	462.0	453.0
15	487.0	497.0	471.0	489.0	506.0	447.0	499.0	467.0	470.0	458.0
16	492.0	493.0	477.0	504.0	508.0	460.0	503.0	471.0	475.0	465.0
17	483.0	494.0	466.0	503.0	497.0	450.0	495.0	461.0	468.0	459.0
18	469.0	483.0	453.0	485.0	485.0	445.0	493.0	449.0	456.0	439.0
19	488.0	484.0	478.0	492.0	516.0	451.0	502.0	445.0	471.0	435.0
20	469.0	457.0	461.0	470.0	479.0	424.0	487.0	421.0	444.0	406.0
21	478.0	461.0	457.0	479.0	465.0	443.0	491.0	431.0	445.0	427.0
22	498.0	495.0	483.0	511.0	480.0	479.0	504.0	458.0	470.0	457.0
23	479.0	529.0	459.0	526.0	474.0	495.0	496.0	472.0	469.0	486.0
Final Week	531.0	549.0	512.0	541.0	518.0	503.0	538.0	496.0	509.0	494.0

FOOTNOTES

1. Weight gain is expressed in g.
2. Indicates control group.
3. Indicates experimental group.

APPENDIX XV

Sign Test for Statistical Comparison of Incidence of Nasal Porphyria in Control and Vitamin E-Deficient Rats

Number	Nasal Porphyria[a] C[b]	E[c]	Sign of $y_1 - y_2$
1	-	+	+
2	-	-	0
3	-	+	+
4	-	+	+
5	-	+	+
6	-	-	0
7	+	-	-
8	+	+	0
9	-	-	0
10	-	+	+
11	-	+	+
12	+	+	0
13	-	+	+
14	-	+	+
15	-	-	0
16	-	-	0
17	-	+	+
18	+	+	0
19	-	-	0
20	+	+	0
			y = 9

a. Plus (+) sign indicates presence of nasal porphyria at time of sacrifice, minus (-) indicates absence of sign.

b. Indicates control group.

c. Indicates experimental group.

Interpretation of Sign Test (Nasal Porphyria)

Null hypothesis: $p = 0.5$ (that is, the distribution of incidence of nasal porphyria for the two groups are identical)

Alternative hypothesis: p differs from 0.5

Test statistic: y, the number of plus signs

Rejection region: Reject null hypothesis if y lies more than 2σ away from μ.

For this data $y_1 = y_2$ for subjects 2, 6, 8, 9, 12, 15, 16, 18, 19 and 20, hence 10 pairs of data are eliminated. Therefore n = 10, p = 0.5 and $\mu = n(0.5) = 10\ (0.5) = 5.0$.

$$\sigma = \sqrt{npq} = \sqrt{10\ (0.5)(0.5)} = 1.58$$
$$2\sigma = 3.16$$

Rejection region will be values of y greater than $(\mu + 2\sigma)$, or 5.0 + 3.16 = 8.16, or less than $(\mu - 2\sigma)$, or 5.0 - 3.16 = 1.84.

Since y = 9 (falls in rejection region) the null hypothesis is rejected and it is concluded that there is a significant difference in the incidence of nasal porphyria.

APPENDIX XVI

Sign Test for Statistical Comparison of Incidence of Greasy-Rough Coat in Control and Vitamin E-Deficient Rats

Number	Greasy-rough coat[a] C[b]	E[c]	Sign of $y_1 - y_2$
1	-	-	0
2	-	+	+
3	-	-	0
4	-	+	+
5	-	+	+
6	-	-	0
7	-	-	0
8	+	-	-
9	-	+	+
10	-	-	0
11	-	+	+
12	-	-	0
13	-	-	0
14	-	+	+
15	-	-	0
16	-	-	0
17	+	-	-
18	-	+	+
19	-	+	+
20	-	-	0
			y = 8

a. Plus (+) sign indicates presence of greasy-rough coat at time of sacrifice, minus (-) sign indicates absence of sign.

b. Indicates control group.

c. Indicates experimental group.

Interpretation of Sign Test (Greasy-rough Coat)

Null hypothesis: $p = 0.5$ (that is, the distribution of incidence of greasy-rough coat for the two groups are identical).

Alternative hypothesis: p differs from 0.5.

Test statistic: y, the number of plus signs.

Rejection region: Reject null hypothesis if y lies more than 2σ away from μ.

For this data, $y_1 = y_2$ for subjects 1, 3, 6, 7, 10, 12, 13, 15, 16 and 20, hence 10 pairs of data are eliminated. Therefore n = 10, p = 0.5 and $\mu = n(0.5) = 10\ (0.5) = 5.0$.

$$\sigma = \sqrt{npq} = \sqrt{10\ (0.5)(0.5)} = 1.58$$

$$2\sigma = 3.16$$

Rejection region will be values of y greater than $(\mu + 2\sigma)$ or 5.0 + 3.16 = 8.16, or less than $(\mu - 2\sigma)$, or 5.0 - 3.16 = 1.84.

Since y = 8 (does not fall in rejection region) it is concluded that there is insufficient evidence to reject the null hypothesis, therefore there is no significant difference in the incidence of greasy-rough coat.

APPENDIX XVII

Criteria for Interpretation of Phospholipid Data

Triplicate values were obtained for all phospholipid samples. With the following exceptions all 3 readings were averaged to determine the final value:

1. When the interpolated value was less than 1 μg (sensitivity of method) that reading was discarded and the lipid was assumed to have deteriorated. If all 3 values were less than 1 μg, no value was reported for that sample.

eg. DPG values for:

+L 1, 20
+L 16, 17
-L 16, 17
+T 16, 17
-T 16, 17
+T 2, 12
+T 10, 19

2. When one of the 3 values was extremely low as compared with the 2 other values that value was discarded. In order for the low value to be eliminated it had to be less than 25% of the mean of the other 2 values. Five readings fell into this category:

eg.

a) DPG +T 7, 15

624
772
141 *eliminated

b) DPG -T 7, 15

382
442
60 *eliminated

c) PE +L 3, 11

```
2568
2520
 495 *eliminated
```

d) DPG -L 3, 11

```
1080
1404
 108 *eliminated
```

e) DPG -L 6, 9

```
1101
 945
 221 *eliminated
```